THE TRANSFER OF ESSENTIAL POLYUNSATURATED FATTY ACIDS FROM THE YOLK TO THE TISSUES DURING AVIAN EMBRYONIC DEVELOPMENT

A thesis submitted to the Faculty of Science
of the University of Glasgow
for the award of the Degree of Doctor of Philosophy

by

André MALDJIAN
S A C
(Scottish Agricultural College)
Biochemical Sciences Department
Auchincruive, Ayr, KA6 5HW

October, 1995.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ACKNOWLEDGEMENTS

I would like to thank the following persons for their immense contributions:

Dr Brian Speake and Dr Raymond Noble, my supervisors, for never ending support, enthusiasm and financial advice throughout these three years.
Kenny MacIsaac for advice on GC, HPLC, computing, folk music.....
Ruth McCartney for her deep knowledge of what could go wrong in lipid analysis.
Dr Silvia Cerolini for her advice and help for the experiments on adipose tissues and analysis of variance.
To all postgraduate students of this department for their sense of humour.
Et finallement, je remercie la Caisse Autonome de Retraite de Médecins Français pour leur support financier qui a permis la réalisation de ce projet.

CONTENTS

ABBREVIATIONS and ENZYME NOMENCLATURE

HPLC	.....	High Performance Liquid Chromatography
HPTLC	.....	High Performance Thin Layer Chromatography
TLC	.....	Thin Layer Chromatography
GC	.....	Gas Chromatograph
et al	.....	and others
IS	.....	Internal Standard
λ	.....	Wave length
min	.....	Minute(s)
sec	.....	Second(s)
ND	.....	Not Detected
w	.....	Weight
w/w	.....	Weight for weight
w/v	.....	Weight for volume
v/v	.....	Volume for volume
S.E.M.	.....	Standard Error of the Mean
psi	.....	pounds per square inch
rpm	.....	revolutions per minute
°C	.....	degrees Celsius
RT	.....	Retention Time
AT	.....	Adipose tissue
BSA	.....	Bovine Serum Albumin
EDTA	.....	Ethylene Diamine Tetra Acetate
IPA	.....	Isopropan-2ol
sn-x	.....	Fatty acid esterified to the x carbon of the glycerol backbone
HDL	.....	High Density Lipoprotein
LDL	.....	Low Density Lipoprotein
VLDL	.....	Very Low Density Lipoprotein
PUFA	.....	Polyunsaturated fatty acid(s)
Cx	.....	Fatty acid(s) with x carbons
CE	.....	Cholesteryl ester
CHOL	.....	Free cholesterol

FFA	Free fatty acid
PL	Phospholipid
TG	Triacylglycerol
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PS	Phosphatidylserine
Sph, SPH	Sphingomyelin
15:0	pentadecaenoic acid
16:0	palmitic acid
16:1	palmitoleic acid (*n-7*)
18:0	stearic acid
18:1	oleic acid (*n-9*)
18:2	linoleic acid (*n-6*)
18:3	linolenic acid (*n-3*)
20:4 or AA	arachidonic acid (*n-6*)
20:5 or EPA	eicosapentaenoic acid (*n-3*)
22:5	docosapentaenoic acid (*n-6*)
22:6 or DHA	docosahexaenoic acid (*n-3*)

ACAT	Acyl-CoA:cholesterol O-acyltransferase (E.C. 2.3.1.26)
Catalase	E.C. 1.11.1.6
CEH	Cholesterol ester hydrolase (E.C. 3.1.1.13)
Cholesterol oxidase	E.C. 1.1.3.6
Desaturases	Δ9-Desaturase (E.C. 1.14.99.5)
Elongase	Acyl COA elongase (E.C. 2.3.1.119)
HL	Hepatic lipase (E.C. 3.1.1.34)
HSL	Hormone sensitive lipase (E.C. 3.1.1.3)
LCAT	Lecithin:cholesterol acyltransferase (E.C. 2.3.1.43)
LPL	Lipoprotein lipase (E.C. 3.1.1.34)
Phospholipase A2	E.C. 3.1.1.4

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

SUMMARY

The quality of the nutrients provided to the developing embryo are of primary importance to ensure the proper functional development of the various organs. The brain and retina require large amounts of certain long chain polyunsaturated fatty acids (PUFA) during embryonic and neonatal life. Docosahexaenoic acid (DHA) is a major component of neural tissues. However, the mechanism of delivery of this fatty acid to the brain still has to be established. The chick embryo was used as model system in the present study. The amounts and proportions of the major fatty acids in the lipids of the yolk, whole embryo, blood plasma, adipose tissue, heart and brain were determined throughout chick embryo development. The results revealed a series of unique features associated with various stages of DHA transport from the yolk to the target tissues. Firstly, DHA was preferentially transferred from the lipids of the yolk complex to the embryo. Secondly, since DHA was present in the initial yolk as a component of phospholipid, but was released into the embryonic circulation in the form of plasma triacylglycerol; it was proposed that DHA is translocated from phospholipid to triacylglycerol in the yolk sac membrane. It was also proposed that the high activity of lipoprotein lipase in the developing adipose tissue promotes the hydrolysis of DHA from plasma triacylglycerol and the incorporation of this DHA into the triacylglycerol of the adipocytes. The pattern of change in the amount of DHA present in adipose triacylglycerol suggested that this fatty acid may be preferentially mobilised from this tissue to be released into the circulation as the free fatty acid. Experiments involving the incubation of adipose tissue pieces *in vitro* also provided evidence for the preferential mobilisation of DHA from the tissue. It was proposed that DHA in the form of plasma free fatty acid is readily taken up by the brain. The amount of DHA in brain phospholipid increased continuously during embryonic development but this increase occurred in parallel with the accretion of the other major fatty acids; i.e. there was no evidence for any preferential incorporation of DHA. Brain phospholipid classes were separated using an HPLC system and it was shown that phosphatidylethanolamine and phosphatidylserine were the major DHA-containing fractions in this tissue. Similar results were also obtained using duck embryo brain. On the basis of the findings of this study, a mechanism for the transfer of DHA from the yolk to brain is proposed; the key feature of this mechanism is the role of adipose tissue as a mediator of this process. The possible relevance of these findings to mammalian and human development is discussed.

LITERATURE REVIEW

It has been argued that the single most important adaptation during the evolution of terrestrial vertebrates was the development of an egg that could sustain the embryo away from the aquatic environment (Romer, 1968). The eggs of reptiles and birds are described as both *amniote* and *cleidoic*. The first term refers to the amniotic sac, within which the embryo develops in an aqueous environment, protected from desiccation. *Cleidoic* refers to the shell that encloses the egg and isolates the embryo from the external environment. Embryonic development within a *cleidoic* egg occurs, therefore, within an essentially closed system in which all the nutrients for the sustenance of the embryo are "pre-packaged" prior to laying, and where only O_2, CO_2 and H_2O-vapour are exchanged with the environment (White, 1991).

The synthesis and deposition of large amounts of nutrients, particularly in the form of yolk, into the egg prior to laying, represents a major metabolic effort by the maternal parent. The mechanisms of this process in the laying hen are described in section *1.1*. In the avian embryo, the early stages of development as reviewed in section *1.2*, proceed with only minimal utilisation of the yolk nutrients. However, the final trimester of avian embryogenesis is characterised by an extremely rapid uptake of yolk components, in which the metabolism of yolk lipids by the embryo assumes paramount importance (Noble and Cocchi, 1990) as described in section *1.3* . The major metabolic functions of the yolk lipids include energy provision to sustain growth and the supply of components for membrane biogenesis. However, a range of more specific functions have also been described including the essentiality of certain polyunsaturated fatty acids, particularly docosahexaenoic acid (22:6; DHA) for the functional maturation of the brain and retina. The importance of DHA during vertebrate development generally is discussed in section *1.4*, and some particular advantages of using the avian embryo as a model system for studying the transport and metabolism of this fatty acid are suggested in section *1.5*. These latter aspects formed the major focus of the present study.

1.1.SYNTHESIS AND ASSEMBLY OF EGG COMPONENTS BY THE PARENT BIRD

1.1.1.Lipids and proteins of the yolk

The colloquial "yolk" of a bird's egg is a single massive cell, the oocyte, in which the cytoplasm proper is mainly restricted to a narrow layer, the germinal disc, which lies adjacent to the plasma membrane at one pole of the cell. By far the major portion of the yolk consists of a mass of lipid-rich droplets and dense proteinaceous granules which form the major nutritive source for the developing embryo. The mechanism of yolk

formation has been described in detail in a series of reviews (Griffin *et al*, 1984; Griffin, 1992; Burley *et al*, 1993; White, 1991; Noble and Cocchi, 1990), and the essential features are described below.

High speed centrifugation of yolk produces 3 fractions : (i) a floating fat layer consisting of triacylglycerol-rich lipoproteins, (ii) a pellet consisting of the granule fraction plus some associated lipid, (iii) an infranatant containing water-soluble proteins. Few, if any, of these components are synthesised in the ovary; the triacylglycerol-rich lipoproteins are initially synthesised by the liver in the form of a specialised type of very low density lipoprotein (VLDL), and the granules originate from a large precursor protein, vitellogenin, which is also synthesised by the liver. The VLDL and vitellogenin are secreted from the liver into the bloodstream, transported to the ovary and incorporated into the developing oocyte. An impressive feature of oocyte development in the ovary is the dramatic growth of the cell, typically from 0.07 to 37.0 mm in diameter in the hen. The uptake of the liver-derived yolk components occurs mainly in the latter phases of this process, during growth from about 4.0 to 37 mm. The ovary at peak performance usually contains at any one time, 5 or 6 oocytes which have attained this stage, in a graded size sequence.

Plasma lipoproteins of the VLDL-type, isolated from the plasma of mammals and birds, have a general common structure, with a core of triacylglycerol and cholesteryl ester and a surface layer of phospholipid, non-esterified cholesterol, and specific apoproteins. The specialised VLDL particles secreted by the liver of the laying bird are unusually small and regular in size, with a mean diameter of 30 ± 5 nm. The much larger VLDL particles present in the plasma of non-laying birds contain 6-9 different apoproteins. In contrast, those of the laying bird contain only 2 apoproteins, namely apoB and apo-VLDL-II. ApoB, the main structural protein of the particle is enormous with a molecular weight of over 500,000 whereas apo-VLDL-II is a much smaller protein of subunit molecular weight 9,500 which exists as a dimer of about 18,000. Hen vitellogenin is a dimer of about 480,000, consisting of 2 identical monomers. It is a highly phosphorylated protein and also contains some associated lipid, mainly phospholipid.

Vitellogenin, apo-VLDL-II, and the specialised "small" VLDL particles are essentially absent from the plasma of male birds, and from immature or non-laying females. With the approach of sexual maturity in the female and the onset of egg laying, a series of dramatic changes take place. The rate of synthesis of lipid from carbohydrate in the liver increases to such a high level that the total concentration of triacylglycerol in the liver increases some 3-fold. The secretion of this lipid results in an increase of some 20-fold in the plasma VLDL concentration.

Moreover, this increase is accounted for entirely by the production of the "small", apo-VLDL-II containing particles typical of the laying bird. Similarly, the

plasma concentrations of vitellogenin, and also some other yolk-specific proteins such as certain vitamin binding proteins, also increase dramatically at this time. All these changes occur in a co-ordinated fashion and are regulated by changes in plasma oestrogen concentration. Thus, hepatic expression of the genes coding for vitellogenin, apo-VLDL-II, vitamin-binding proteins, and lipogenic enzymes are induced or enhanced by oestrogen. The expression of these genes can also be induced in male and immature female birds by administration of oestrogen.

The VLDL particles produced by the laying bird possess properties which promote their delivery to the ovary and uptake into the oocyte. In particular the small size of these particles is apparently a modification to facilitate uptake into the oocyte. In the ovarian follicle, the growing oocyte is surrounded by several layers of supporting tissue. Blood capillaries permeate the outer, thecal layer. These capillaries are exceptionally permeable, enabling large particles such as lipoproteins to diffuse through to the next layer, the basal lamina. This is a connective tissue layer that surrounds the oocyte and acts as a filter, preventing the passage of large lipoproteins such as the intestinal portomicrons but permitting the transfer of the smaller apo-VLDL-II containing lipoproteins. The yolk precursors then pass between the granulosa cells which surround the oocyte. The binding of the VLDL particles to specific receptors on the oocyte plasma membrane is followed by receptor-mediated endocytosis and the delivery of the yolk precursors into the oocyte. The surface area of the oocyte is greatly enlarged by extensive convolutions of the plasma membrane, in order to promote the efficient bulk transfer of yolk precursors into the cell. The binding of the VLDL to the receptor occurs via the apo-B component and not via the apo-VLDL-II. However, the receptor is distinct from the LDL receptor present on other cell types. Interestingly, vitellogenin and VLDL are taken up by the oocyte via the same receptor. Birds with the *restricted ovulator* mutation lack this receptor on the oocytes and consequently accumulate VLDL and vitellogenin in the plasma as opposed to deposition in the yolk.

Following uptake into the oocyte, the protein components of the yolk precursors are proteolytically modified. Apo-B is cleaved to give a series of smaller proteins, the apovitellenins III, IV, V and VI, whereas the apo-VLDL-II subunit remains intact and is referred to as apovitellenin I. Vitellogenin is cleaved to give two proteins, phosvitin (molecular weight approximately 30,000) and lipovitellin (molecular weight approximately 340,000). 54 % of the amino acids of phosvitin are serine residues, with 95 % of these phosphorylated. Thus phosvitin accounts for 80 % of the protein-bound phosphorus of the yolk whereas lipovitellin contains 15-20 % associated lipid. Following uptake, the endocytotic vesicles packed with yolk precursor particles undergo a series of fusions to form large, membrane bound yolk spheres. These consist of tightly packed lipoprotein particles plus granules consisting of aggregated phosvitin-lipovitellin complexes.

In contrast to the modifications to the protein components, the lipids of the VLDL are deposited in the yolk essentially unaltered. Thus the fatty acid composition of the yolk lipid will reflect that of the VLDL lipid assembled in the liver. Dietary fat cannot be directly incorporated into the yolk since the portomicrons released from the intestine are too large to cross the basal lamina. The fatty acids incorporated into VLDL by the liver may be derived by hepatic lipogenesis from carbohydrate precursors, uptake of portomicron remnants, or mobilisation of adipose tissue triacylglycerol stores. The latter may be derived from a combination of *de novo* synthesis and dietary sources. Whereas the saturated and monounsaturated fatty acids of the VLDL may be derived from both *de novo* lipogenesis from carbohydrate and from the diet, the polyunsaturated components can only be provided from the diet. Thus the DHA content of the yolk lipid can only be derived from dietary fatty acid, either in the final form, or as the linolenic acid precursor. A number of studies (Anderson *et al*, 1989; Cherian and Sim, 1992, 1993) have shown that feeding of oils enriched in either DHA or linolenic acid to the parent bird enhances the DHA content of the yolk lipid. The biosynthetic and / or dietary origins of the major fatty acids present in animals are outlined in Figure 1.1.

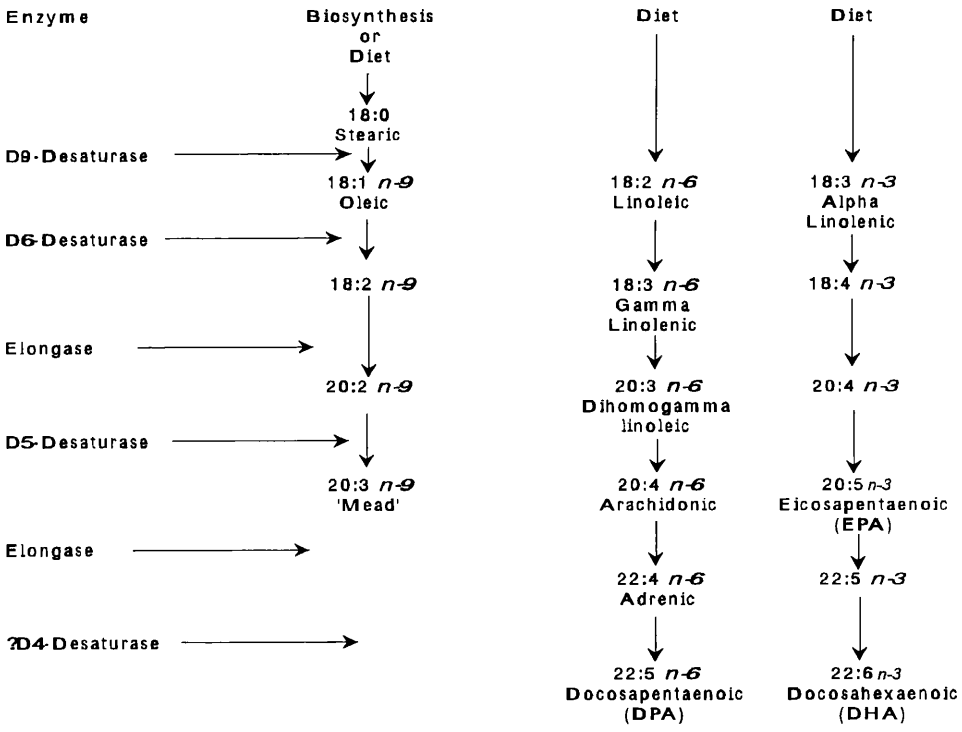


Figure 1.1: The separate biosynthetic pathways for the 3 families of fatty acid share and compete for the same enzymes. Thus Δ6-desaturase can use either 18:1 n-9, 18:2 n-6 or 18:3 n-3 as substrate. The conversion of 18:1 n-9 through to 20:3 n-9 is usually only favoured when 18:2 n-6 and / or 18:3 n-3 are deficient. Likewise the synthesis of 22:4 n-6 and 22:5 n-6 often only occurs when n-3 fatty acids are deficient.

1.1.2. Other yolk components

The protein content of the aqueous phase of the yolk are collectively known as livetins and are derived from the plasma. Livetins α-, β-, and γ- correspond respectively to serum albumin, α_2-glycoprotein and γ-globulin (Burley *et al*, 1993; Griffin *et al*, 1984).

In addition to lipid and protein for energy and growth, the embryo also needs to be supplied with all the vitamins, minerals and trace elements needed for development. Binding proteins for riboflavin, biotin and thiamin are synthesised in the parental liver in response to oestrogen stimulation and serve to transport these vitamins to the oocyte. The binding proteins and their associated vitamins accumulate in the aqueous phase of the yolk (Griffin *et al*, 1984; White, 1991). Vitamin A is present in the aqueous phase as a complex with retinol-binding protein and pre-albumen, and an identical complex is responsible for the plasma transport of this vitamin to the ovary (Griffin *et al*, 1984; Vieira and Schneider, 1993). Vitamin D is transported in the plasma and deposited in the yolk in association with a cholecalciferol-binding protein (Griffin *et al*, 1984). Vitamin E is probably supplied to the yolk as a component of the VLDL (Cohn *et al*, 1992).

The high phosphate content of vitellogenin/phosvitin enables it to bind large amounts of Ca^{2+},and also Mg^{2+} and Fe^{3+}. These and various other metal ions are consequently found complexed to the granule fraction (Griffin *et al*, 1984).

1.1.3. Formation of albumen, shell membranes and shell

In the hen, a single ovary and oviduct are located on the left hand side of the body; the respective organs on the right hand side having undergone degeneration during development. In a laying hen, oocytes of all sizes are present in the ovary, from microscopic to the fully formed yolk-rich ovum ready to escape from the follicle. Ovulation occurs at approximately daily intervals, when the ovum (i.e. the "yolk") escapes by rupture of the follicle and is picked up by the *infundibulum* (mouth) of the oviduct. The ovum is driven down the oviduct by peristaltic contractions. Fertilisation occurs in the upper part of the oviduct and the early cleavage divisions of the zygote occur before laying (Romanoff, 1960).

The egg remains in the *infundibulum* for 15-30 min and then spends the next 2-3 hours passing down the portion of the oviduct called the *magnum.* Albumen proteins are synthesised and secreted by specialised cells of the endometrial glands of the magnum. The oviductal expression of the genes coding for the major albumen proteins (ovalbumen, ovotransferrin, ovomucoid, lysozyme and avidin) is induced by oestrogen.

This hormone also promotes the differentiation of the endometrial glands in immature chicks. Thus the synthesis both of the yolk components by the liver and albumen components by the oviduct is coordinated by oestrogen. The twisting movement of the egg as it passes along the *magnum* result in the formation of layers of the albumen around the yolk (Palmer and Guillette, 1991). From the *magnum*, the egg enters the *isthmus* portion of the oviduct where it becomes surrounded by the shell membranes. These consist of a network of proteinaceous fibres secreted by the endometrial glands of the *isthmus* (Palmer and Guillette, 1991). The egg passes from the *isthmus* to the *shell gland*, where during the 4.5-24 hour period post-ovulation, mineral secretion and crystallisation occurs producing a shell consisting mainly of calcite (Board and Sparks, 1991)

1.1.4. Composition of the newly-laid egg

In an average newly-laid egg weighing approximately 60g, four principal parts may be described : the shell (6g), the shell membranes and air space, the albumen (34g) and the yolk (20g). The germinal disc or blastoderm which gives rise to the embryo and its associated membranes "floats" on the upper surface of the yolk (Romanoff, 1960).

The calcite shell has a well-defined system of pores which permit the exchange of respiratory gases and also the loss of a proportion of the egg's water content during incubation. A thin proteinaceous cuticle prevents bacterial penetration via the pores (Board and Sparks, 1991).

The albumen consists of 4 % (w/w) of solid matter in an aqueous environment. The solid matter is largely made up of proteins but as many of these are glycosylated there is also a significant carbohydrate content. At least 13 proteins have been identified with the major one, ovalbumen, making up 54% (w/w) of the total. Others include lysozyme, ovomucoid and ovotransferrin. Albumen provides a mechanical support for the yolk and also exerts a major antimicrobial function. The albumen is ingested orally by the embryo from about day 12 of development and therefore represents an important nutritional protein resource for the embryo (Palmer and Guillette, 1991).

Yolk consists of approximately 50 % (w/w) solids and 50 % water. Lipids make up about 66 % (w/w) of the solid matter, proteins about 33 %, and only a very small proportion of carbohydrate (Romanoff, 1960). Thus the average 60g egg contains about 6g of lipid. The majority of yolk lipid (> 90 %) is associated with the VLDL-derived fraction. The concentrations of the yolk major individual lipid fractions with their fatty acid composition are given in Table 1.1.

The data in this table are based on eggs from parents which have received a normal commercial diet.

Table 1.1 : Yolk lipid and fatty acid composition before incubation (Noble and Cocchi, 1990).

	CE*	TG*	PE*	PS*	PC*	Sph*	FC*
W. % of total lipid	1.3	63.1	7.1	0.81	20.5	0.30	4.9
Fatty acids, w. % of total							
16:0	29.1	24.5	21.7	33.6	33.7	41.7	
16:1	1.0	6.6	1.1	5.4	1.0	6.5	
18:0	9.5	6.4	30.1	27.3	15.8	17.6	
18:1	40.1	46.2	15.3	15.9	27.7	23.7	
18:2	18.0	14.7	9.2	7.3	14.1	9.1	
18:3	0.3	1.1	<0.5	<0.5	<0.5	<0.5	
20:4	0.9	0.3	13.2	8.5	4.4	<0.5	
22:6	0.5	<0.5	8.4	1.2	1.8	<0.5	

*CE=cholesteryl ester; TG=triacylglycerol; PE=phosphatidylethanolamine; PS=phosphatidylcholine; Sph=sphingomyelin; FC=free cholesterol

The principal lipid fraction is triacylglycerol which is expected considering the high rate of synthesis of this fraction by the liver of the laying hen (see *1.1.*). The second major component is the phospholipid fraction and the free cholesterol is the third. Oleic acid is the major fatty acid in the cholesteryl ester, triacylglycerol and total phospholipid fractions. Phospholipid fractions have substantial levels of long chain polyunsaturated fatty acids (arachidonic and docosahexaenoic acid).

This yolk composition is susceptible to modifications if the hen is under conditions of nutritional or environmental stress (Noble and Cocchi, 1990).

1.2.DEVELOPMENT OF THE CHICK EMBRYO

1.2.1.Fertilisation

The ovum of a bird is a single cell, but because of the large amount of yolk, the cell membrane is supplemented by the vitelline membrane for support. Fertilisation occurs in the *infundibulum* soon after ovulation, when the ovum interacts with sperm which have migrated up the oviduct. The sperm penetrate the vitelline membrane,

facilitated by the release of hydrolytic enzymes from the acrosome. In birds, many sperm penetrate the vitelline membrane but only one fertilises the egg (Bellairs, 1992).

Recent studies suggest that lipids have important roles in sperm function. In most mammals (Lin *et al*, 1993) sperm phospholipids contain exceptionally high level of DHA, and there is evidence that adequate levels of this fatty acid are essential for maximal motility and fertility. However, chicken sperm appears to be deficient in DHA and instead contain high levels of 22:4 *n-6* (Darin-Bennett *et al*, 1974). In mammalian sperm, the release of diacylglycerides and long-chain polyunsaturated fatty acids from membrane phospholipids appears to be a key stage in the signalling cascade which triggers the acrosome reaction (Roldan and Hamilton, 1993).

1.2.2. Early stages of development

The cleavage divisions of the fertilised egg occur during passage down the oviduct. Because of the large yolk mass, cell division is restricted to the protoplasmic portion of the egg where the zygote nucleus is located. The daughter nuclei are separated by infoldings of the plasma membrane which eventually fuse to delineate the cells, forming a circular blastodisc initially one cell layer thick and later several cells thick. At the time of laying, the blastodisc consists of about 60,000 cells and is 2.5-5.8 mm in diameter. Between 9 and 20 h after laying a condensation of cells known as the primitive streak forms along the central line of the blastodisc. A gastrulation process then occurs as cells of the blastodisc migrate to the streak and pass through it to become the mesodermal and endodermal layers, whilst those remaining at the surface form the ectoderm. By about one day of incubation, an infolding of the ectoderm along the mid-line begins to produce the neural tube, the precursor of the central nervous system. The formation of the neural tube proceeds from anterior to posterior. By 36 h, the neural tube in the anterior region has closed to form 3 brain vesicles. Thus the ectoderm furnishes all the neural elements of the body. The early development of the embryo is described in detail in several articles (Romanoff, 1960; Bellairs, 1991, 1992; Slack, 1991). The role of inductive interactions between the primary cell layers in the differentiation of specific structures such as the neural elements is discussed by Slack (1991). Shortly after the beginning of incubation the activity of the *Hox* (homeobox) genes in controlling the development of particular embryonic regions and the formation of the basic body pattern becomes apparent, and there is some evidence that gradients of retinoic acid may partly regulate this expression (Bellairs, 1992). Morphogenic gradients of retinoic acid also appear to regulate the pattern of digit formation in the developing limb buds (Ragsdale and Brockes, 1991).

Although the blastoderm of the newly laid egg contains about 60,000 cells only about 500 will contribute to the embryo proper, the rest forming extraembryonic structures including the amnion, chorio-allantois, and yolk sac membrane (Slack, 1991). A major role for the yolk sac membrane is that of yolk lipid uptake and transfer and its development has been described by Noble and Cocchi (1990). This structure grows outwards from the embryo, spreading over the yolk surface to eventually envelop the entire yolk. The yolk sac membrane is divisible into 2 major structural areas : an outer mesoderm of flattened cells to form a supportive membrane and an inner endoderm of columnar cells which are responsible for yolk absorption. From about the fourth day of development, the yolk sac membrane begins to develop markedly with the formation of elaborate folds and a microvillus structure. At the same time, the yolk sac membrane undergoes extensive vascularisation through the development of a network of capillaries emanating from within the villi. The blood flowing from these capillaries passes into the portal system of the embryo via several major vitelline veins which enter the embryo through the yolk stalk. There is no evidence of any lymphatic system within the yolk sac membrane. Although anatomically the yolk sac membrane is essentially an extension of the embryo's hind gut, passage of yolk material directly into the intestine is prevented by a loop of tissue.

1.2.3 The embryo as a physiological system

The nutrition of the embryo during the early stages of development is poorly understood but it has been claimed that glucose and amino acids form the major energy source at this time. However, during the latter half of development which represents the major growth period, the energy metabolism of the embryo is overwhelmingly dominated by the utilisation of yolk lipid. In fact, the β-oxidation of fatty acids derived from yolk lipid provides approximately 95 % of the energy needs of the embryo over the whole developmental period (Romanoff, 1967; Freeman and Vince, 1974; Noble and Cocchi, 1990). Consideration of the physiological and biochemical processes occurring within the egg from about the half way stage of development reveals a coordinated series of complex functions designed for the delivery of nutrients from the yolk to the embryo, the utilisation of additional nutrients from the albumen and shell, the exchange of respiratory gases, and the disposal of waste products. The extraembryonic membranes play a key role in these inter-relationships. The role of the yolk sac membrane in the transfer of lipids and also proteins, vitamins and minerals from the yolk to the embryo has already been mentioned (section *1.2.2*). Gaseous exchange occurs through the pores of the calcite shell and transport of O_2 and CO_2 between the shell and the interior is mediated by the blood vessels of the highly vascularised chorioallantoic

membrane which lies beneath the shell membranes and which therefore fulfills the functions of a lung. The rates of O_2 uptake and CO_2 release by the egg increase dramatically from about day 10, reaching a plateau level by day 18 of incubation. These respiration characteristics largely reflect the rates of β-oxidation of yolk derived lipids by the embryonic tissues (Romanoff, 1967; Freeman and Vince, 1974). A second major role of the chorioallantoic membrane is the mobilisation of the calcium of the shell to supply the needs of the embryo. The mechanism of this process involves the conversion of respiratory CO_2 to carbonic acid by the action of carbonic anhydrase, the solubilisation of shell calcium by this acid and the translocation of this calcium into the circulation involving the action of a chorioallantoic Ca^{2+} binding protein (Tuan *et al*, 1987).

The newly laid egg does not contain a major store of carbohydrate. Thus the synthesis of glucose by the gluconeogenic pathway, probably using amino acids as precursors, assumes a major importance in the embryo. The key gluconeogenic enzymes are expressed in the embryo's liver from around the 7th day and increase to a maximal value by day 17. Much of the gluconeogenic effort is directed towards glycogen production, and both the liver and the yolk sac membrane are major sites of glycogen storage (Freeman and Vince, 1974).

1.2.4. The hatching process

The major features of hatching have been described by Freeman and Vince (1974). The onset of pulmonary respiration probably starts from the time the beak penetrates the inner shell membrane and enters the air space (internal pipping). It has been suggested that increases in the arterial concentration of CO_2 act as a stimulus for this process. During the period of approximately 25 h between internal pipping and hatching, the responsibility for respiration is gradually switched from the chorioallantois to the lungs.

Immediately prior to the initiation of breathing there is a profound mobilisation of glycogen stores from both the liver and the yolk sac membrane, stimulated by glucagon and catecholamines. The adipose tissue of the embryo also develops sensitivity to the lipolytic effects of glucagon during the hatching period. Thus mobilisation of the stores of both lipid and carbohydrate accumulated by the embryo appear to contribute to the energy needs of hatching.

Some 9 h after internal pipping, the beak fractures the shell over the air space. This external pipping process is apparently stimulated by a rise of pCO_2 in the air space and is facilitated by the previous thinning of the shell during calcium mobilisation.

From about Day 19 of incubation, the yolk sac and its residual yolk content are progressively withdrawn into the abdominal cavity. There is evidence that yolk sac retraction is stimulated by thyroid hormones (Decuypere *et al*, 1991). The nutrient content of the retracted yolk sac is sufficient to maintain the hatchling for several days. The chick emerges from the shell some 16 h after external pipping. O_2 consumption increases during this 16 h period, and then increases more dramatically in the 10 h after emergence.

1.3.TRANSPORT AND METABOLISM OF LIPIDS IN THE CHICK EMBRYO

1.3.1.Introduction

The almost total reliance on yolk lipids for energy metabolism, in addition to the provision of lipid components for cell membrane biogenesis and the existence of more specific roles such as the essentiality of DHA for adequate neural development, emphasises the crucial role of lipid metabolism in avian embryo development. In general terms the utilisation of yolk lipids by the embryo can be described by the following sequence of events : (i) the uptake and processing of yolk lipids by the yolk sac membrane; (ii) the assembly, within the yolk sac membrane, of the yolk derived lipid component into lipoproteins particles; (iii) the release of these lipoproteins into the circulation and their delivery to the embryo's tissues via the bloodstream; (iv) the uptake and metabolism of the lipid components of the lipoproteins by the tissues. However, within this overall scheme there is also a requirement for the specific delivery, or targeting, of particular lipid moieties, to the different tissues at defined stages of development; e.g. the specific incorporation of DHA by the brain and retina, and the delivery of cholesterol to the gonads and adrenals for steroid hormone synthesis. Early studies by Noble and Moore (1967a) showing that the liver lipids at day 12 of development consisted of triacylglycerol containing up to 20 % DHA, phospholipid containing more than 20 % arachidonic acid and cholesteryl ester containing up to 80 % oleate (i.e. in drastic contrast to the original yolk lipid composition, see Table 1.1; percentages refer to w/w of total fatty acid acids) provided a dramatic illustration that the lipid utilisation process did not simply consist of bulk transfer and non-specific β-oxidation, but instead involved major tissue-specific rearrangements of the proportions and compositions of the lipid classes. It was suggested (Noble and Cocchi, 1990) that the high level of DHA in liver triacylglycerol was somehow related to the mechanism and route of transfer of this fatty acid from the yolk to the neural tissues, and more recent studies (Speake *et al*, 1993) lend support to this view.

1.3.2. Uptake and processing of yolk lipid by the yolk sac membrane

The yolk sac membrane of the avian embryo is in some respects analogous to the mammalian placenta in its role in the provision of nutrients to the embryo, although some of the aspects of lipid processing and secretion show some comparability with the function of the small intestine in dietary lipid uptake. However neither of these analogies provide an accurate picture of yolk sac membrane function, which exhibits a range of unique and characteristic features.

The mean weights of total lipid associated with the yolk contents, yolk sac membrane and tissue of the chick embryo at various stage of incubation are shown in Figure 1.2.

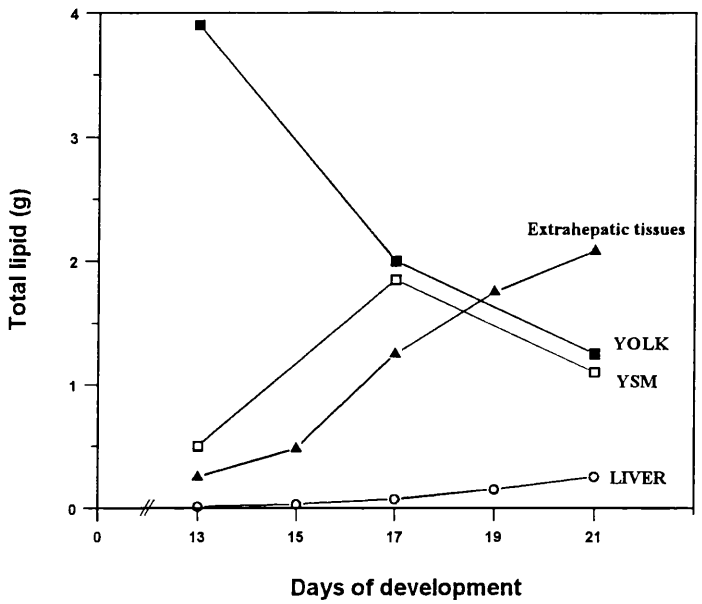


Figure 1.2 The relative movement of lipid from the yolk contents into the chick embryo via the yolk sac membrane. Weights of total lipid in the yolk contents, yolk sac membrane and embryonic tissues during the last week of incubation (Noble and Cocchi, 1990).

Net lipid loss from the yolk of a 60 g egg during the first 13 days of development only amounts to a couple of hundreds of mg (Table 1.2). However, between days 13 and 21, the loss of lipid increases substantially such that during the last two days of incubation, the yolk lipid is removed at its highest rate. The amount of yolk lipid decreases from around 4 g at day 13 to 1 g at day 21 (hatching), i.e. an overall loss of 400 mg per day. A preferential absorption of lipid relative to other components from

the yolk contents over this period is indicated by a decrease in the percentage of total lipid in the dry matter, from 65% on day 13 of incubation to 54 and 44% respectively on day 15 and 21. Between days 13 and 17 of incubation, loss of lipid from the yolk contents is accompanied by its rapid accumulation in the yolk sac membrane, such that by day 17 the membrane contains as much lipid as the contents (Noble and Moore, 1967b). Just prior to hatching, there is a rapid loss of lipid from the membrane, from 82% at day 17 to 68% at day 21 (% w/w of lipid in its dry matter; Noble and Cocchi, 1990).

Table 1.2: Lipid changes in the yolk during incubation (Noble and Cocchi, 1990).

	Yolk content		
Day of incubation	0	13	21
Total lipid, g	6.11	4.93	0.80
Lipid composition, w % of total			
CE	1.3	0.42	0.50
CHOL	4.9	6.11	5.00
TG	63.1	71.8	75.9
PL	29.7	21.4	18.2
Phospholipid composition, w % of total			
PE	23.9	14.0	8.30
PS	2.7	1.21	3.43
PC	69.1	75.4	71.7
Sph	1.0	1.79	2.67

Within the phospholipid fraction of the yolk there is a preferential removal of phosphatidylethanolamine (PE) relative to the other major fractions (Noble and Moore, 1967b). Thus whereas the PC/PE ratio of the unincubated egg yolk is about 3.0, it rises to around 8.6 in the yolk contents by day 21 of incubation. The weights of PC in the yolk decreases by about 74 % (from about 1.25 g to 0.32 g) from day 0 to day 17 while that of PE decreases by some 90 % (from 433 mg to 44 mg) (Table 1.2).

Morphological and biochemical evidence has established that the yolk lipid uptake by the yolk sac membrane occurs through non specific phagocytosis. Plates 1a and 1b present two pictures from electron microscopy showing the sequential engulfment of intact lipid droplets and their appearance at the apical surface of the

Plates 1a and 1b show electron microscopic pictures of the yolk sac membrane at day 15 of incubation showing engulfment of the yolk material by the microvilli (see Noble and Cocchi, 1990). Plate 1a: yolk interface at the top of the picture. Plate 1b: yolk lipid droplets (dark vesicles) being taken up by the yolk sac membrane microvilli.

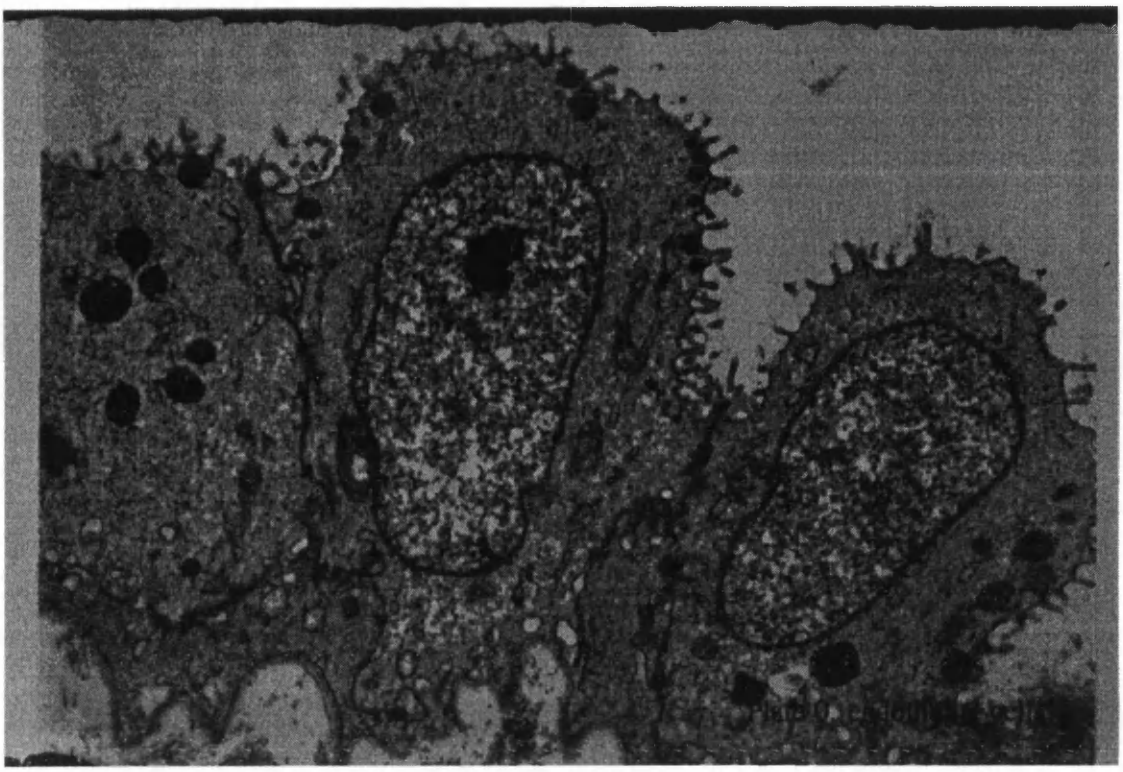


Plate 1a

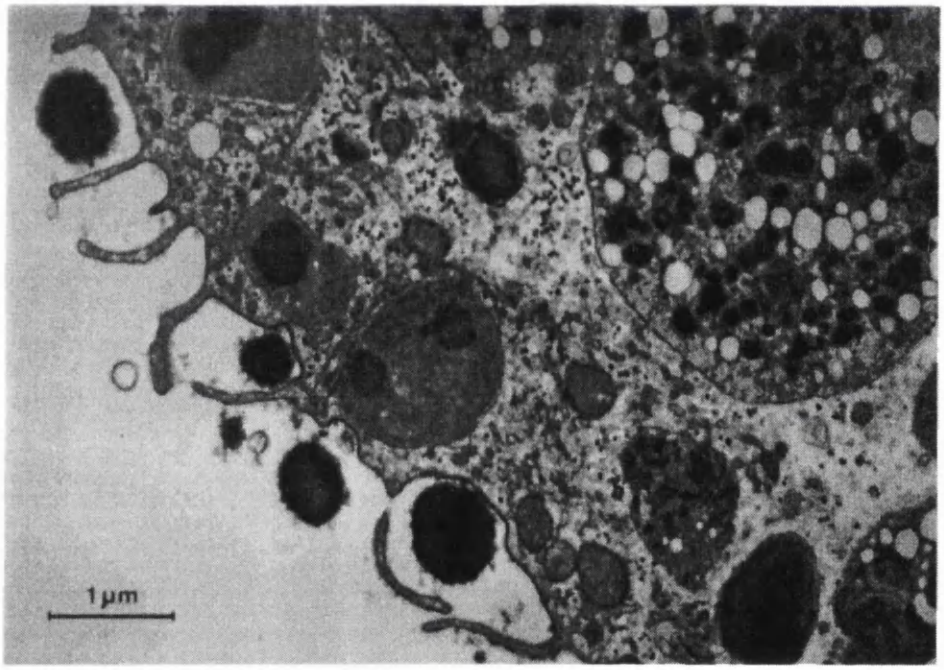


Plate 1b

endodermal cells. Yolk lipid droplets are trapped by the apical microvilli (Figure 1.3) and they are then enclosed within apical vesicles. These vesicles containing the absorbed lipid are transferred away from the apical surface to the basal surface where as a result of merger during transfer, they display physically different properties. Intracellular lipid droplets have been identified at quite early stages of development (6th day of incubation), their level increasing until about the 14th day onwards they form the principal inclusion of the endodermal cell (Noble *et al*, 1988).

There is no real evidence for extracellular digestion playing a part in the uptake of the major lipids into the membrane. Very low levels of lipid breakdown products (e.g. free fatty acids, partial glycerides) are found within the yolk during the most intense period of lipid uptake. However, there is considerable evidence that yolk lipid undergoes a range of modifications and re-arrangements within the yolk sac membrane. Perhaps the most drastic modification concerns the esterification of yolk-derived cholesterol, within the yolk sac membrane, to form cholesteryl ester. Whereas approximately 80 % of yolk cholesterol is present as the unesterified sterol, with only 20 % as the ester, these proportions are effectively reversed (Table 1.3) within the yolk sac membrane where over 80 % of the total cholesterol content is present as the esterified form (Shand *et al*, 1993). Very high levels of acylCoA:cholesterol acyltransferase (ACAT), the enzyme responsible for cholesterol esterification, were detected in the microsomal fraction of the yolk sac membrane throughout the latter half of the developmental period (Shand *et al*, 1993).

Table 1.3 : Lipid composition of yolk and yolk sac membrane at day 14 of development. Each lipid class is expressed as percentage (w/w) of total lipid. Total lipid content is expressed as percentage (w/w) of wet tissue. $^{b}P<0.001$, significant differences (Student's t-test) between lipid compositions of yolk and yolk sac membrane.

Lipid class	Yolk	Yolk sac membrane
Cholesterol	5.4 ± 0.2^{b}	1.3 ± 0.1
Cholesteryl ester	1.3 ± 0.1^{b}	5.6 ± 0.3
Triacylglycerol	67.4 ± 0.4	67.8 ± 1.2
Phospholipid	25.2 ± 0.3	23.8 ± 1.1
Free fatty acid	0.7 ± 0.1	1.1 ± 0.1
Total lipid content	31.3 ± 0.2	30.8 ± 0.9

The activity of ACAT, measured using the cholesterol endogenous to the microsomes as substrate, was already high at day 9 of incubation, but this value increased nearly 3-fold by day 16 and then decreased by day 20. The levels of ACAT activity correlated with the rate of uptake of yolk lipid by the yolk sac membrane.

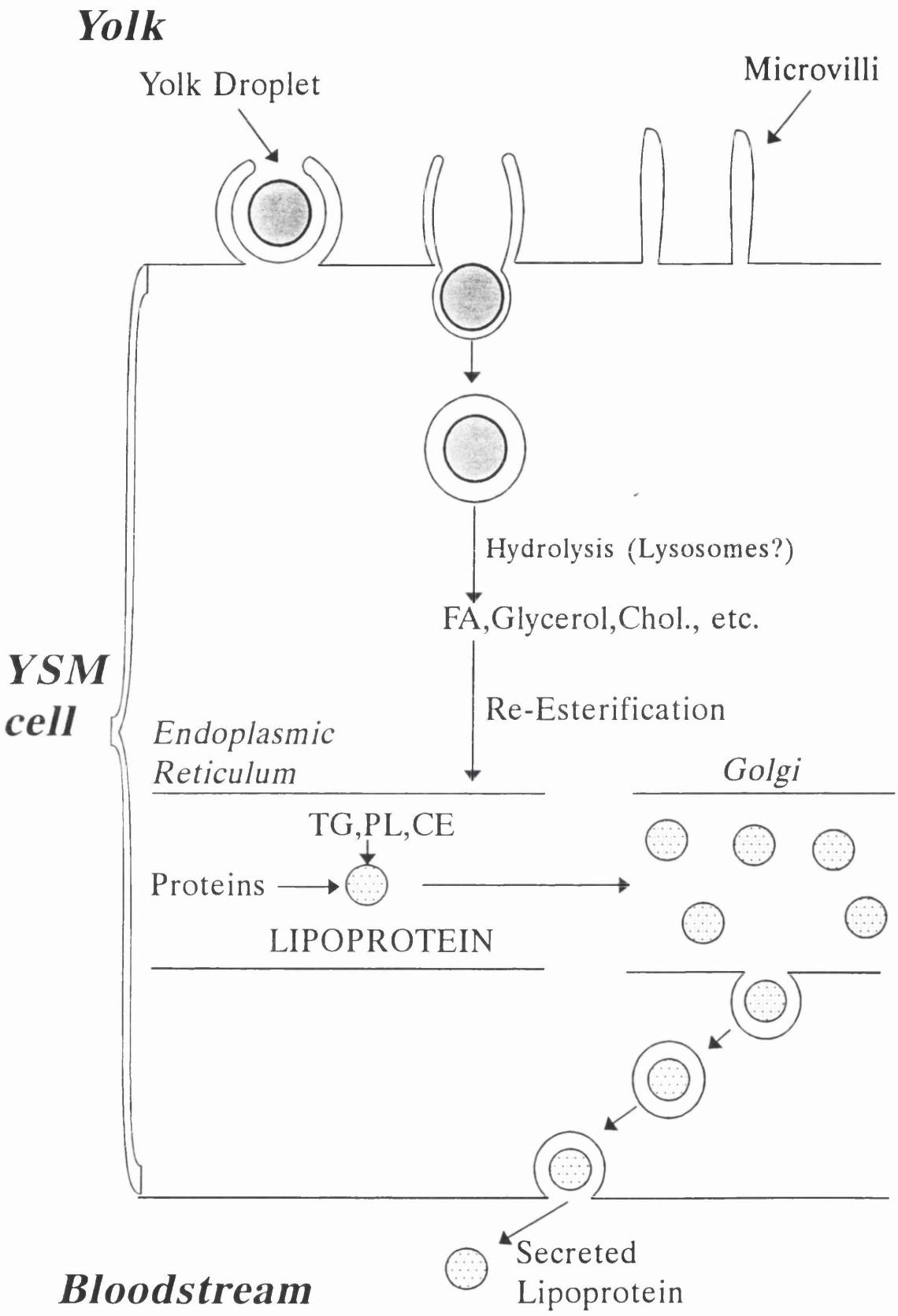


Figure 1.3 : Proposed mechanism of yolk lipid assimilation by the yolk sac membrane.

Furthermore, Reinhart *et al* (1993) demonstrated the presence of a protein in the yolk sac membrane which is similar to mammalian sterol carrier protein (SCP_2); this protein is implicated in the transport of sterols through the cytoplasm and is known to stimulate ACAT. The activity of ACAT was increased by the addition of exogenous cholesterol to the microsomes with the greatest stimulation obtained at day 9 and 12, while less stimulation was observed for the later stages of development (Table 1.3). This suggests that the enzyme in the microsomes may be approaching saturation with substrate as development processes. This view was supported in the same study (Shand *et al*, 1993) by the fact that the concentration of endogenous microsomal cholesterol increased almost 4 fold between days 9 and 20 of development. In fact, ACAT activity was found to be highly correlated with the microsomal cholesterol concentration between days 9 and 16 but poorly correlated prior to hatching at day 20. On the basis of these studies, Shand *et al* (1993) suggested that the increased potential for cholesterol esterification in the yolk sac membrane between days 9 and 12, and the decrease between days 16 and 20, were due primarily to alterations in the total level of ACAT present, whereas the increase between days 12 and 16 was due predominantly to increased levels of cholesterol substrate in the microsomes (Table 1.4).

Table 1.4 : ACAT activities in microsomes isolated from yolk sac membranes of embryos during development. Exogenous cholesterol added to give a final concentration of 250 μg/ml. $^{b}P<0.001$; $^{c}P<0.01$ (Shand et al, 1993).

	Cholesteryl oleate formed (pmol/min/mg protein)	
Days of development	Endogenous cholesterol	Exogenous cholesterol
9	326 ± 42^{b}	696 ± 72^{c}
12	550 ± 71^{b}	1100 ± 115
14	720 ± 57^{c}	1023 ± 77
16	929 ± 56	1162 ± 49
20	592 ± 13^{b}	693 ± 40^{c}

The activities of ACAT within the yolk sac membrane are high in comparison with previously published values for mammalian tissues, i.e. activities in mammary gland, heart, brain and pancreas are generally less than 1 % of the activity observed in the yolk sac membrane at day 16 (Shand and West, 1991 a,b). Even mammalian tissues which are actively involved in cholesterol metabolism such as liver, intestine, ovary and adrenal cortex, typically express levels of ACAT which are less than 20 % of those reported for the yolk sac membrane (Billheimer *et al*, 1981; Erickson *et al*, 1980; Cadigan *et al*, 1988). Furthermore the activity of cholesteryl ester hydrolase (CEH), an enzyme with the potential to reverse the effects of ACAT, within the yolk sac membrane

is less than 2 % of the maximum ACAT activity throughout the developmental period. High levels of a cytosolic CEH-inhibitor protein are also present in this tissue (Shand *et al*, 1993). All these factors are a combination well suited to promote an essentially unidirectional conversion of cholesterol to cholesteryl ester. Thus, Shand *et al* (1993) concluded that the relevant enzymic systems in the yolk sac membrane are very strongly directed towards cholesterol esterification, with very little propensity to reverse this process. It should also be noted that the cholesteryl ester synthesised in the yolk sac membrane was predominantly in the form of cholesteryl oleate (Table 1.5). It appears that the ACAT system in this tissue may have a high specificity for oleoyl CoA, or alternatively oleate may be the major acyl group provided to the esterification system (Shand *et al,* 1993).

Analyses of the yolk contents during incubation, have shown that within the phospholipid fraction there is a preferential removal of phosphatidylethanolamine species enriched in DHA (Noble and Moore, 1967b). Thus there is a preferential uptake of DHA by the yolk sac membrane. In addition, the proportion of arachidonic acid in the phospholipids of the yolk sac membrane is much greater than in the yolk, due to the conversion of linoleic to arachidonic acid by the action of the Δ6 desaturase and other enzymes in this tissue (Noble and Cocchi, 1990). Table 1.5 illustrates the proportional increase of polyunsaturated fatty acids within phospholipid classes between yolk and yolk sac membrane.

Table 1.5 : Fatty acid composition (w percent of total) of the Cholesteryl Ester, Phosphatidylethanolamine and Phosphatidylcholine fractions of the yolk contents and yolk sac membrane at day 21 of incubation (Noble and Moore, 1967 b).

	CE		PE		PC	
	Yolk content	Yolk sac membrane	Yolk content	Yolk sac membrane	Yolk content	Yolk sac membrane
16:0	11.7	4.27	25.7	13.7	32.2	28.2
16:1	2.71	1.77	1.04	1.19	1.08	1.32
18:0	3.67	3.71	26.5	36.6	16.6	18.3
18:1	55.9	75.9	25.9	13.4	29.3	27.8
18:2	21.8	12.9	12.0	10.8	16.4	16.6
18:3	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
20:4	1.26	0.63	5.07	15.7	2.68	4.35
22:6	2.46	0.30	1.79	6.44	1.14	2.55

Both the incorporation of oleate into cholesteryl ester and the conversion of linoleic to arachidonic acid described above, will require the prior hydrolysis of fatty

acids from the complex lipids derived from the yolk. In addition, the electron microscopy studies of Lambson (1970) provided evidence that extensive modifications of the lipid droplets occur in the yolk sac membrane, resulting in the re-modelling of the contents to form lipoprotein particles which are secreted by exocytosis from the basal surface of the endodermal cells. Taken together, the evidence suggests that the lipids taken up from the yolk may be extensively hydrolysed in the yolk sac membrane, possibly by lysosomal action, followed by re-esterification at the endoplasmic reticulum and packaging into lipoprotein particles which enter secretory system (i.e. endoplasmic reticular lumen, Golgi, secretory vesicles) of the cell. This scheme is illustrated in Figure 1.3.

1.3.3.Assembly and release of plasma lipoprotein by the yolk sac membrane

Evidence from electron microscopy (Plate 1a) has demonstrated that lipoprotein particles are secreted from the basal surface of the yolk sac membrane endodermal cells into the adjacent capillary system. Thus, via the extensive vascular system of the yolk sac membrane, it may be envisaged that these lipoproteins will enter the embryo's circulation system.

The rapid conversion of yolk-derived cholesterol to cholesteryl ester may be an obligatory stage in the assembly of stable lipoproteins (Griffin, 1992; Shand *et al*, 1993). Cholesteryl esters are synthesised by ACAT at the cytoplasmic face of the endoplasmic reticulum. They are extremely hydrophobic and have been shown to accumulate initially between the leaflets of the endoplasmic reticulum bilayer (Olofsson *et al*, 1987). Studies using hepatocytes have indicated that nascent apo-B, the major structural apoprotein, binds co-translationally to the luminal leaflet of the same subcellular organelle, and it has been proposed that it is only released into the cisternal space following interaction with cholesteryl ester (Olofsson *et al*, 1993). Thus it appears that the assembly of stable lipoprotein particles may be directly linked to the production of cholesteryl ester, and therefore ACAT activity, suggesting that in the yolk sac membrane, cholesterol esterification may be an important determinant of lipid transfer to the embryo. Additional studies using hepatocytes have also suggested that triacylglycerol synthesis in the microsomes may also have an important role in the early stages of lipoprotein assembly (Boren *et al*, 1993). The synthesis of triacylglycerols from yolk-derived acyl components in the yolk sac membrane and their assembly into the embryonic lipoprotein have yet to be investigated. It is likely that the lipoprotein released from the yolk sac membrane will be enriched in both DHA and arachidonic acid due to the selective uptake of some phospholipid classes within the yolk and Δ6-desaturation in the yolk sac membrane as described above. Yolk lipid absorption is associated with increasing

lipid levels within the plasma of the embryo. Between days 13-18 of incubation, the plasma lipid concentration increases continuously with a slight fall occurring just prior to hatching (Yafei and Noble, 1990). The increased lipid level is largely accounted for initially by an accumulation of triacylglycerol-rich very low density lipoprotein. However, as incubation proceeds, the level of triacylglycerol in this lipoprotein fraction is decreased and that of cholesteryl ester increased, such that just prior to hatching, cholesteryl ester accounts for more than half of the total lipid present (Yafei and Noble, 1990).

1.3.4. Utilisation of plasma lipid by the tissues. The role of Lipoprotein lipase

In adult vertebrates, triacylglycerol-rich plasma lipoproteins from dietary or hepatic sources are processed by the action of lipoprotein lipase. This enzyme is present at the luminal surface of the blood capillaries of specific tissues where it catalyses the hydrolysis of triacylglycerols present in plasma chylomicrons and very low density lipoproteins. The fatty acids thus released can be taken up and utilised by the adjacent tissues (Cryer, 1987). The presence of this enzyme in specific tissues of the chick embryo would be consistent with the operation of a similar mechanism for the utilisation of the triacylglycerol rich lipoproteins released by the yolk sac membrane, and this possibility was investigated by Speake *et al* (1993). Table 1.6 shows the lipase activities found in several different organs.

Table 1.6 : Lipase activity characteristics of chick embryo tissues at day 14 of incubation (Speake et al, 1993).

	Lipase activity (units/g tissue)		
	Standard assay	+NaCl	-apoC-II
Adipose tissue	87.4 ± 0.3	3.4 ± 0.3	7.1 ± 0.2
Heart	30.2 ± 0.4	2.5 ± 0.2	2.1 ± 0.1
Thigh muscle	5.8 ± 0.2	2.1 ± 0.1	2.0 ± 0.1
Breast muscle	1.3 ± 0.2	0.8 ± 0.1	0.8 ± 0.1
Liver	6.0 ± 0.1	5.5 ± 0.1	4.6 ± 0.3
Brain	4.6 ± 0.2	4.7 ± 0.2	4.5 ± 0.1

Lipoprotein lipase is characterised and identified by its sensitivity to inhibition by NaCl and its requirement for apoC-II as an activator (Olivecrona and Bengtsson-Olivecrona, 1987). Thus the fact that the activities expressed in extracts of adipose tissue and heart were inhibited by the presence of NaCl or the absence of apoC-II by

more than 90 % confirmed the identity of the enzyme as lipoprotein lipase. It is clear from these results that the activity of the enzyme in adipose tissue is exceptionally high. Although some lipase activity was exhibited by extracts of liver and brain in the assay, little or no reduction in this activity was brought about by the addition of NaCl or the omission of apoC-II. It would therefore appear that lipoprotein lipase is absent from liver and brain, with one or more lipases being responsible for the activities expressed in the assay. It was suggested by Speake *et al* (1993) that the lipase activity in the liver may be due to the expression of hepatic lipase, an enzyme which is located in vascular system of the liver and which functions in the metabolism of lipoprotein remnants. The lack of lipoprotein lipase in the brain may be of particular relevance when considering the mechanism of DHA uptake by this tissue.

The study of Speake *et al* (1993) indicated that the initial expression of lipoprotein lipase activity in adipose tissue, and indeed the early development of the tissue itself, was observed at around day 12 of incubation coincident with the beginning of intensive yolk lipid uptake. Furthermore, it was shown that the activity of the enzyme in heart also increased at this time. Thus it appears that the transfer of lipid from the yolk to the circulation, and the induction of lipoprotein lipase are coordinately regulated. The inducing stimulus is, as yet, unknown, although increased plasma levels of certain hormones occur at this stage (Freeman and Vince, 1974; Scanes *et al*, 1987).

It is likely that the growth of adipose tissue during chick embryo development is almost entirely a consequence of lipoprotein-mediated uptake of plasma lipid, since the rate of lipogenesis from carbohydrate precursors is extremely low (Goodridge, 1973). Moreover the mobilisation of fatty acids from the tissue is very limited until the hatching period (Langslow, 1973), suggesting that the amount of adipose tissue lipid during embryo development may be a reflection simply of the functional activity of lipoprotein lipase in the tissue and the plasma concentration of its lipoprotein substrate (Speake *et al*, 1993). The activity of lipoprotein lipase in adipose tissue decreased transiently over the hatching period, then increased dramatically with the onset of feeding (Speake *et al*, 1993).

An earlier study by Langslow (1972) indicated that the lipolytic sensitivity of chicken adipose tissue to glucagon increases dramatically over the hatching period. It was shown that this hormone stimulates glycogen mobilisation from the liver and fatty acid mobilisation from adipose tissue at this time.

The composition of the tissue lipids of the chick embryo is striking in that the proportion of long-chain polyunsaturated fatty acids therein is very high in comparison with the original yolk contents. The phospholipids of the liver and heart are enriched in arachidonic acid whereas those of the brain and retina are especially high in DHA (Noble and Cocchi, 1990). Furthermore the triacylglycerols of adipose tissue, heart and liver are notable for the presence of high levels of DHA, a feature which is not exhibited

by the yolk contents, and which is unusual in tissues of terrestrial adult birds and mammals (Speake *et al*, 1993).

Studies of the lipid compositions of individual tissues of the chick embryo during development show a range of unique features when compared with comparable tissues from other animal species. The most extreme of these tissue lipid changes is displayed by the liver. Noble (1986) showed that 5 % (w/w) of the lipid taken up from the yolk by the embryo is accumulated by the liver by day 19 of incubation. However, as can be seen in Table 1.7, its composition bears no resemblance to that of the lipid absorbed from the yolk.

Table 1.7 : Lipid and fatty acid changes in the liver during the last week of incubation (Noble and Cocchi, 1990).

	CE		TG		PL	
Day of incubation	13	21	13	21	13	21
Lipid concentration, mg per liver	1.63	122.0	1.07	5.39	3.63	21.5
Lipid distribution, %	23.3	80.2	15.1	3.58	51.4	14.2
Fatty acid composition, wt % of total						
16:0	2.31	1.95	23.4	23.0	22.4	17.8
16:1	0.91	0.97	1.41	1.52	<0.5	<0.5
18:0	2.04	2.74	8.81	11.1	24.6	28.4
18:1	77.2	79.6	30.4	35.7	7.29	7.97
18:2	12.7	12.5	8.32	8.23	9.94	13.8
18:3	<0.5	<0.5	<0.5	<0.5	1.21	1.07
20:4	2.24	1.47	3.52	2.92	26.2	20.0
22:6	1.91	0.42	20.1	11.6	6.45	9.12

The accumulation of lipid in liver during the last week of development is almost wholly accounted for by cholesteryl ester which represents 80% of the total lipid of the liver and 30% of the dry matter (Noble and Moore, 1967a). The triacylglycerol and phospholipid which normally predominate in any liver tissue barely rise in concentration and by the time of hatching amount to only 1 and 7%, respectively, of the total lipid present. A further feature of the accumulated cholesteryl esters is their extremely high content of oleic acid.

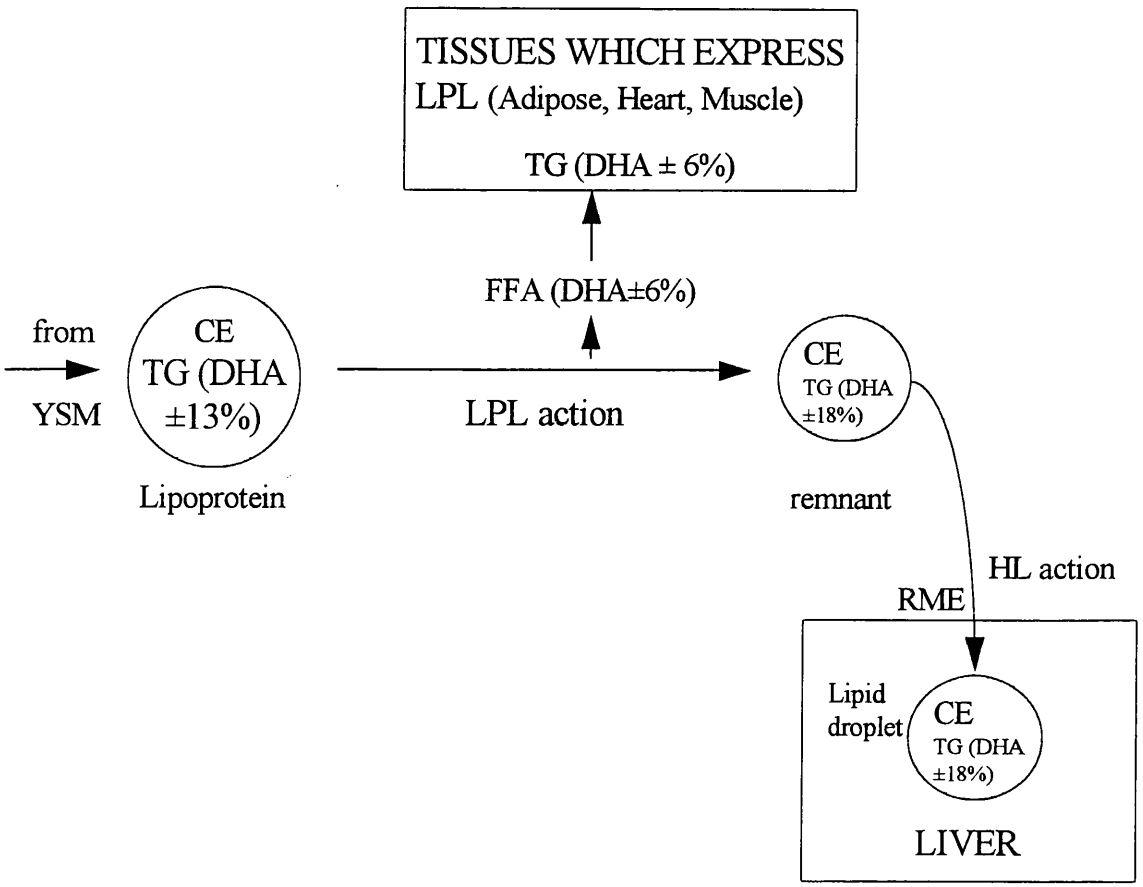
Table 1.7 also shows that during embryonic development the fatty acid composition of the liver phospholipids are characteristically different from that of the yolk as they display considerably more stearic, arachidonic and docosahexaenoic acids

and considerably less palmitic and oleic acids (Noble and Cocchi, 1990). These characteristic differences are a reflection of both differences in the proportions of the various phospholipid moieties and in their fatty acid compositions. Phosphatidylcholine is the major phospholid class: 55% of the total at day 13 but decreasing towards the end of incubation; while the proportion of phosphatidylethanolamine and phosphatidylserine remains constant at about 30 and 12% respectively (Noble and Cocchi, 1990). The two last phospholipid fractions display very much higher levels of arachidonic and docosahexaenoic acids (Noble and Moore, 1967a) than in the yolk during the last week of incubation. The most pronounced changes in the fatty acid composition of the liver phospholipids during embryo development occur in the phosphatidylcholine fraction and involve alterations to both saturated and unsaturated acids (Noble and Moore, 1967a). These analyses show that at some stage during the transfer from yolk to embryo, the phospholipids undergo extensive breakdown and that new phospholipids are synthesised in the embryonic tissues.

The surprising feature of the triacylglycerols found within the liver of the embryo during incubation is the very high concentration of DHA, as this fatty acid is more commonly associated with phospholipids (Noble and Cocchi, 1990). At day 13 of incubation, the level of DHA in the triacylglycerol fraction of the liver is considerably greater than in the phospholipids. Speake *et al* (1993) proposed a theory about the role of lipoprotein lipase in tissue specific lipid uptake (Figure 1.4) which would explain tissue lipid compositions.

The intense level of lipid catabolism via β-oxidation is associated with a significant accumulation of ketone bodies in the plasma. Rapid changes occur in the lipid oxidative abilities of several major tissues, e.g. heart and liver, during the second half of the incubation period (Noble *et al*, 1986). However such changes may not necessarily be solely due to alterations in oxidizable lipid substrate supply; in the case of the liver, the specific apolar nature of the unusual lipid accumulation may affect subcellular function. The requirements and mechanisms of the oxidative processes, which can be observed from about the 8th day of incubation, are similar to those for fatty acid oxidation in mammalian tissues (Koerker and Fritz, 1970). The extent of lipid oxidation can be measured by the rapid increase in the concentration of carbon dioxide within the enlarging air space that develops latterly between the inner and outer shell membranes; thus, whereas at day 13 of incubation, carbon dioxide accounts for only about 1.7% of the total gas present in the air space, just prior to hatching the carbon dioxide concentration exceeds 5% (Freeman and Vince, 1974, Tullett and Noble, 1988).

Figure 1.4 : Proposed model for the role of lipoprotein lipase in tissue-specific lipid uptake. This hypothesis attempts to explain tissue lipid compositions in terms of the distribution and substrate specificity of lipoprotein lipase. Lipoproteins released into the embryonic circulation from the yolk sac membrane are initially processed by the action of lipoprotein lipase located in the capillaries of adipose tissue, muscle and heart. The proportion of 22:6 in the fatty acids thus released is less than originally present in the lipoprotein triacylglycerol due to the relative resistance of esters of this fatty acid to hydrolysis by lipoprotein lipase. This reduced proportion of 22:6 in the free fatty acids available for tissue uptake and re-esterification is reflected in the composition of triacylglycerol deposited in adipose tissue, etc. The action of lipoprotein lipase results in the conversion of the lipoproteins to smaller remnants consisting mainly of cholesteryl ester. The residual triacylglycerols of the remnant are enriched in 22:6, again due to the relative resistance of esters of this fatty acid to lipoprotein lipase. Remnant uptake by the liver results in the accumulation in this tissue of triacylglycerols rich in 22:6 as well as cholesteryl ester. YSM : yolk sac membrane; TG : triacylglycerol; FFA : free fatty acid; CE : cholesteryl ester; LPL : lipoprotein lipase; HL : hepatic lipase; RME : receptor-mediated endocytosis. The proportions (% w/w of total fatty acids) of 22:6 in triacylglycerol and free fatty acid fractions are indicated. These are approximate values for the early lipid uptake period. The values for the free fatty acid and remnant are assumed to be equivalent to those for adipose and liver triacylglycerol, respectively (Speake et al, 1993).


TISSUES WHICH EXPRESS
LPL (Adipose, Heart, Muscle)
TG (DHA ± 6%)
FFA (DHA±6%)
from
YSM
CE
TG (DHA
±13%)
Lipoprotein
LPL action
CE
TG (DHA
±18%)
remnant
HL action
RME
Lipid
droplet
CE
TG (DHA
±18%)
LIVER

1.3.5.Deposition of cholesterol ester in embryo's liver

It may be envisaged that the action of lipoprotein lipase on the lipoproteins released from the yolk sac membrane, will deplete the lipoproteins of their triacylglycerol content, resulting in the formation of much smaller remnant particles consisting largely of cholesteryl ester (Figure 1.4). By analogy with other system (Goldstein and Brown, 1977) it may be proposed that the remnant lipoprotein will be taken up by the liver, by a receptor-mediated endocytotic process. The deposition of large amount of cholesteryl ester in the embryo's liver (Table 1.7) would be consistent with such mechanism.

The preponderance of cholesteryl oleate in the liver sterol esters (Table 1.7) is consistent with the view that the lipid deposited in the liver originates from that assembled in the yolk sac membrane (Noble and Cocchi, 1990). Cytoplasmic lipid droplets are discernible in the liver at day 13 of incubation but they are few in number at this time and similar in size to the adjacent mitochondria. However by day 19 a marked accumulation of lipid occurs in the liver cells, with large lipid droplets occupying the bulk of the cytoplasm. In fact, between days 12 and 20, the amount of cholesteryl ester in the liver increased 37-fold, when expressed on the basis of tissue fresh weight (Shand *et al*, 1994). The study of Shand *et al* (1994) indicated that the ACAT activity in the liver follows the same pattern as that displayed in the yolk sac membrane: i.e. an increase to a maximal value at day 16 followed by a decrease by day 20 of incubation. The activities of the enzyme in the liver were markedly lower (by approx. 4-fold) than those in the yolk sac membrane. Work in other systems has shown that receptor-mediated uptake of lipoprotein remnants by the liver is followed by lysosomal hydrolysis and the release of free cholesterol and fatty acids, initially into the cytoplasm (Goldstein and Brown, 1977). The presence of significant levels of ACAT activity in the liver of the chick embryo (Shand *et al*, 1994) would appear to provide a potential mechanism for the rapid re-esterification of this cholesterol, and its subsequent storage as droplets of cholesteryl ester in the cytoplasm.

The activity of microsomal cholesteryl ester hydrolase in the chick embryo was very low, and even at its maximal value (day 20) represented only 25% of the activity found in the liver of the adult rat (Shand *et al*, 1994). This activity will be decreased by the action of the CEH-inhibitor protein present at all stages; the embryonic liver seems therefore to display very little capacity for the mobilisation of the accumulated cholesteryl ester (Shand *et al*, 1994).

Although there is no definitive evidence for any mechanism by which the embryo may reduce the hepatic accumulation of cholesteryl esters, excretion through the bile into the small intestine may be one possible pathway as the liver lipids of chick embryo show accumulation of cholesteryl oleate at the same time of the appearance of

substantial quantities of this component within the bile (Noble and Cocchi, 1990). Similarly, the presence of high levels of arachidonic acid in the bile phospholipids parallels the accumulation in the liver of phospholipids with unusually high levels of arachidonic acid. Subsequent to the liver and bile changes, an increase has been observed in the ratio of esterified:free cholesterol in the yolk content (Noble and Moore, 1967b), the occurrence of which could not be explained by any association with the metabolic changes in the yolk sac membrane. Also, following the injection of ^{14}C-cholesterol into the yolk, autoradiographic analysis over the incubation period has detected substantial and increasing levels of radioactivity within the gall bladder and gastrointestinal tract of the embryo (Jain *et al*, 1972). Sufficient evidence exists, therefore, to suggest that, during the latter part of development, the bile may provide a limited means of homeostatic control over the lipid accumulation in the liver (Noble and Cocchi, 1990).

1.3.6.Lipid metabolism and the emerged chick

About a quarter of the lipid that was originally present in the yolk (1-2 g) remains unabsorbed at the time of hatching when the yolk sac membrane and its residual contents are retracted into the body cavity (Noble and Moore, 1964; Noble and Moore, 1967b). Following hatching, there is a rapid uptake of the residual yolk material from the retracted yolk sac such that by the 5th or 6th day after hatching, only a vestigial amount remains (Entenman *et al*, 1940, Noble and Ogunyemi, 1989, Romanoff, 1960). The lipid metabolism of the newly hatched chick undergoes a series of rapid changes. The massive stores of cholesteryl ester in the liver are dramatically depleted within a few days after hatching and, in the absence of a supply of yolk lipid, the liver develops the enzymic capacity to synthesise fatty acids and triacylglycerol from dietary carbohydrate (Noble and Cocchi, 1990).

1.4. THE ROLE OF *N-3* POLYUNSATURATED FATTY ACIDS DURING VERTEBRATE DEVELOPMENT

In many animal cell types, polyunsaturated fatty acids of both the *n-6* and *n-3* series have a number of important roles, mainly related to the structure and function of cell membranes and the production of eicosanoids. An adequate provision of essential polyunsaturates is especially important during early development, since DHA in particular is required by the brain and retina during critical periods of differentiation.

1.4.1. General effects of DHA on embryonic growth and survival

n-3 fatty acids appear to be essential for the survival of embryos and emergent larvae of certain fish species. The consistent occurrence of high levels of these fatty acids in the lipids of fish eggs, and their preferential incorporation into membrane phospholipids as opposed to oxidation for energy, suggests that relatively large amounts of *n-3* polyunsaturates may be necessary during the early stages of fish development. Deficiency of *n-3* fatty acids in the yolk lipid of trout embryos was associated with high levels of embryonic mortality, and adequate levels of these fatty acids were required to support normal growth rates in embryos and larvae of a range of fish species (Henderson and Tocher, 1987).

Current evidence also suggests that an adequate supply of DHA may be essential for the survival of alligator embryos. Reduced levels of DHA in the yolk lipids of eggs from alligators currently reared in captivity were associated with a high degree of embryonic mortality in comparison with eggs from wild alligator (Noble *et al*, 1993).

The effects of DHA-deficiency during embryogenesis of the higher vertebrates may be less drastic. In a study by Anderson *et al* (1989), reduced levels of *n-3* fatty acids in the yolk lipids of chicken eggs resulted in greatly decreased levels of DHA in the brain of the newly-hatched bird but no increase in embryo mortality. Similarly, in mammals DHA-deficiency is not normally associated with increased embryonic mortality (Neuringer *et al*, 1988). In humans, maternal dietary intakes of arachidonic acid and DHA were positively correlated with placental weight, birthweight and head circumference (Crawford *et al*, 1989)

1.4.2. DHA and retinal function

Very high levels of DHA are present in the photoreceptor cells of the retina. Rod photoreceptor outer segments consist of stacks of disks, each composed of a pair of membrane bilayers. These membranes have an unusually low cholesterol content (<10 mol % of total lipid) and an exceptionally high content of phospholipid (80-90 mol %). These phospholipids contain extremely high levels of polyunsaturated fatty acids, with DHA comprising up to 65% of the total fatty acids of phosphatidylethanolamine and phosphatidylserine. In the 2-position of these phospholipids, DHA accounts for 75-100% of the total fatty acids. The 1-position also contains up to 25% DHA, resulting in the formation of suprenoic molecular species with DHA in both positions. Rod outer segment phosphatidylcholine also contains significant levels of very long chain polyunsaturated fatty acids containing 24-36 carbon atoms (Neuringer *et al*, 1988).

The presence of polyunsaturated fatty acids in membrane phospholipids produces increased membrane fluidity, compressibility, and permeability. Although DHA with six double bonds, is unique in its high degree of polyunsaturation, its incorporation into membrane phospholipids does not necessarily result in high fluidities. Studies using artificial phospholipid membranes with different fatty acids in the 2-position have shown that the presence of DHA compared to 18:2 *n-6* does not cause a further increase in fluidity (Coolbear *et al*, 1983). Thus DHA's role in retinal function may involve properties other than fluidity, such as the physical flexibility or compressibility of the membrane.

The rod outer segment is responsible for transducing photons to electrical signals. This process is mediated by rhodopsin, an integral membrane protein which accounts for 90% of the protein in the disk membrane. Activation of rhodopsin results in a major conformational change which must be accommodated by the membrane bilayer. It has been proposed that the presence of DHA in phospholipids results in a membrane that is relatively thin but is able to expand in response to rhodopsin activation. The basis of this property may be related to the postulated ability of DHA in the membrane to undergo a transition between helical and uncoiled conformations (Dratz *et al*, 1987; Neuringer *et al*, 1988; Sanders, 1988). It has also been suggested that the high DHA content may increase the permeability of the disk to ions involved in neural signalling (Sanders, 1988).

1.4.3. DHA and brain development

In cerebral grey matter, DHA makes up approximately 30% of the fatty acid content of phosphatidylethanolamine and phosphatidylserine. The highest levels of DHA are present in synaptosomes, synaptic vesicles, mitochondria and microsomes. In contrast, white matter and its myelin fraction contain very little DHA (Neuringer *et al*, 1988).

Brain development in cellular terms, consists of a sequence of events including neuronal multiplication followed by cell stretching and neurite extension, synapse formation and myelination. The timing of these processes differs among mammalian species. In rats, mice and rabbits, myelination commences 10-15 days after birth and is completed several months later, whereas in the guinea pig myelination is largely completed prior to birth. In humans and primates, myelination occurs both pre- and post-natally. DHA accumulation in the neurones largely occurs in the phase immediately prior to myelination, and is associated with the periods of neurite extension and axonal formation. Thus in the rat, the level of DHA in the brain increases rapidly between birth and 16 days of age whereas in the guinea pig DHA accumulation is largely

pre-natal. During human brain development the DHA content increases 3-5 times in the last trimester of foetal life, and a further 3-5 times during the first 12 post-natal weeks. Similarly, in rhesus monkeys, the percentage of DHA in the cerebral cortex at birth is approximately half the adult value (Neuringer *et al*, 1988; Burdge and Postle, 1994; Kamazawa *et al*, 1991; Davison, 1965).

The precise role of DHA in brain development remains to be ascertained. The timing of DHA accumulation is consistent with the incorporation of this fatty acid into the membranes of maturing neurones prior to, and in support of, neurite extension and axon formation (Burdge and Postle, 1994). Addition of DHA to neuronal cell cultures stimulates neurite extension (Burdge, G.C., personal communication) and has also been reported to promote neuronal cell division (Bourre *et al*, 1983). The period of most rapid accumulation of DHA generally precedes the onset of electrical activity and the expression of neurite growth cone associated protein (Burdge and Postle, 1994). The presence of high levels of DHA in the excitable membranes of the nervous system may affect a range of parameters including the biophysical and structural properties of the membrane-bound enzymes associated with neurotransmission, membrane permeability, and the synthesis of eicosanoids and docosanoids (Neuringer *et al*, 1988).

1.4.4. Consequences of DHA deprivation during development on neural and retinal function

The incorporation of DHA into neural lipids at a defined stage of differentiation suggests that an adequate provision of this fatty acid is specifically required during a particular developmental period. Consequently, a number of studies have been undertaken to investigate the possibility that an inadequate DHA supply during the developmental period may lead to irreversible impairments of neural and retinal function. The major conclusions of these studies have been described in reviews by Neuringer *et al* (1988) and Sanders (1988) and are discussed below.

Initial studies in which rats were fed diets deficient in *n-3* but rich in *n-6* fatty acids indicated that the brain and retina were very resistant to DHA depletion compared to other tissues. Furthermore, the brain subfractions which normally contain high levels of DHA, such as synaptosomes, showed less proportionate DHA depletion than oligodendrocytes, myelin and peripheral nerves, which normally have low DHA levels. Dietary depletion for 2 or more generations reduced DHA levels in the brain and retina to 8-20 % of control values. These decreases in DHA were compensated by increases in 22:5*n-6* and to a lesser extent 22:4*n-6* so that the total level of C22-polyunsaturated fatty acids remained almost constant, and the degree of polyunsaturation decreased only slightly. Normally, 22:5*n-6* and 22:4*n-6* are present at very low levels in animal tissues,

and their appearance is an indication of DHA deficiency. It is possible that these fatty acids are able to fulfill, at least partially, some of the roles of DHA and their synthesis may represent a homeostatic mechanism for maintaining neural membrane function. DHA-deficient rats were, however, found to exhibit a degree of visual impairment as quantified by electroretinogram studies on the physiological response evoked from the retina by brief flashes of light. Such rats also suffered to some extent from behavioural impairments, as reflected in reduced exploratory behaviour and in maze-learning tasks.

Similar studies on non-human primates have also been described (Neuringer *et al*, 1988; Sanders, 1988). Feeding of diets low in *n-3* but high in *n-6* fatty acids to rhesus monkeys throughout pregnancy resulted in offspring in which, at the time of birth, DHA levels were reduced to 25% of control in the brain and 50% of control in the retina. Continuation of this *n-3* deficient diet to the mothers during suckling prevented any further increase in neural DHA levels, in contrast to controls where the levels doubled. By 2 years of age, DHA levels in both tissues were 15-20 % of normal. As in rats, there was a quantitative substitution of 22:5*n-6* for DHA. Visual acuity and electroretinogram function were found to be substantially impaired in the DHA-deficient monkeys, although tests on behavioural ability were less conclusive. The reversibility of *n-3* deficiency was studied in 10-24 month old monkeys, after the deficiency was well established and brain growth was virtually complete. Supplementation of the diet with *n-3* fatty acids resulted in an increase in cerebral DHA to control levels within 12 weeks, while levels of 22:5*n-6* declined. However, the visual acuity and electroretinogram impairments did not improve, suggesting that DHA-deficiency during early development irreversibly alters retinal function.

These studies on rodents and primates have been confirmed and extended by more recent investigations (Wainwright *et al*, 1991; Kanazawa *et al*, 1991; Enslen *et al*, 1991). Until recently, information regarding humans has been limited, although Holman *et al* (1982) described a case of *n-3* deficiency in a 6-year old child characterised by peripheral neuropathy and blurred vision. Evidence has, however, been accumulating over the last few years emphasising the importance of adequate DHA provision for the human foetus and neonate. Studies on premature infants have indicated that the ability to synthesise DHA from 18:3*n-3* is inadequate to meet the demands of the nervous system (Carlson *et al*, 1987; Uauy *et al*, 1990). Thus DHA must be provided by the mother to the foetus or neonate respectively via the placenta or the milk. Indeed Sinclair (1975) has shown that, in rats, DHA is incorporated into the brain at 10-times the rate of 18:3*n-3*. Post-mortem analysis of brain lipid composition in victims of Sudden Infant Death Syndrome indicated that infants who had been fed artificial bottle milk lacking in DHA exhibited deficiencies in brain DHA compared to breast-fed babies (Farquharson *et al*, 1992). Furthermore, a study on the effects of post-natal feeding on premature babies found that those infants who received breast milk had significantly

higher IQ's at 8 year of age compared to those who were bottle fed (Lucas *et al*, 1992), and it was suggested that these effects were related to DHA provision. The importance of an adequate supply of DHA to the human foetus and neonate has been stressed in a recent report by the British Nutrition Foundations' Task Force (1992).

1.4.5. The route and mechanism of DHA-delivery to the neural tissues.

In species such as the rat, where DHA accumulation is restricted to the post-natal period, polyunsaturated fatty acids are obtained from milk lipids during suckling. In contrast, in the guinea pig where neuronal differentiation is essentially pre-natal, polyunsaturated fatty acids are delivered to the foetus via the placenta. In the human, where DHA accumulation in the brain occurs both pre- and post-natally, delivery of polyunsaturated fatty acids will initially be a placental and later a lactational function. However the mechanisms whereby DHA and other long chain polyunsaturates are delivered to the developing brain, either pre- or post-natally, are not known (Neuringer *et al*, 1988). Several mechanisms have been proposed regarding the form in which polyunsaturated fatty acids are taken up by the brain. On the basis of a series of detailed studies, Dhopeshwarkar and Mead (1973) suggested that plasma free fatty acids bound to albumin (or α-foetoprotein) are the preferred source, and a report by Anderson and Connor (1988) lent support to this view. In contrast, recent evidence that cells of the central nervous system express receptors for a range of lipoproteins (Pitas *et al*, 1987; Handelman *et al*, 1992) raises the possibility of neural uptake of polyunsaturates as components of plasma lipoproteins. It is of interest that receptor-mediated uptake of lipoproteins by foetal rabbit brain cells was found to promote neurite growth, extension and branching (Handelman *et al*, 1992). An alternative mechanism of DHA supply to the neonatal brain was proposed by Scott and Bazan (1989) on the basis of investigations using suckling rats. These studies suggested that dietary DHA from the milk is initially incorporated into phospholipids of the neonate's liver, and then secreted as the phospholipid component of very low density lipoprotein for delivery to the brain. A similar role for the neonatal liver as a mediator of DHA transport to the retina was proposed by Li *et al* (1992). However the means by which, in this scheme, DHA could be transferred from the VLDL phospholipid to the neural cells is not clear.

With regard to possible mechanisms of delivery of DHA to the foetal brain, the role of the placenta in the supply of lipid components from the maternal to the foetal circulation assumes paramount importance. In most species studied, it is clear that free fatty acids from the maternal plasma are able to traverse the placenta and enter the foetal circulation (Noble and Shand, 1981; Noble and Cocchi, 1989). In addition, the hydrolysis of maternal plasma triacylglycerol by placental lipoprotein lipase also results

in the transfer of the derived free fatty acids into the foetal circulation (Noble and Shand, 1981; Faulkner and Jones, 1979). This transfer of free fatty acid across the placenta appears to be mediated by a plasma membrane fatty acid binding protein (Dutta-Roy, personal communication). In many species, the concentrations of arachidonic acid, DHA and other polyunsaturates in the foetal circulation are significantly higher than in the maternal circulation, and the existence of selective transfer and desaturation/elongation mechanisms have been demonstrated (Noble and Cocchi, 1989).

The delivery of DHA across the placenta in the free fatty acid form into the foetal circulation would be compatible with the possibility of subsequent uptake of this non-esterified DHA by the foetal brain in line with the views of Dhopeshwarkar and Mead (1973). It has been suggested, at least in the case of the guinea pig, that the foetal liver is not involved in the mediation of this transfer (Burdge and Postle, 1994), raising the likelihood that DHA is transported, in the free fatty acid form, direct from the placenta to the foetal brain. The period of DHA acquisition by the foetal and neonatal brain is accompanied by dramatic changes in the maternal hepatic DHA metabolism and the concentrations of DHA in the maternal plasma lipids in rats (Hunt *et al*, 1991), humans (Postle *et al*, 1994) and guinea pigs (Burdge and Postle, 1994).

1.5.THE CHICK EMBRYO AS A MODEL FOR *N-3* FATTY ACID METABOLISM

The chick embryo presents several advantages as a model system for studying DHA transport to the neural tissues during development. Yolk lipids are the primary nutritive source supporting embryogenesis and the embryo exists essentially as a closed system within the egg utilising the pre-packaged nutrients. Consequently there is a temporal separation of the maternal and embryonic phases of lipid translocation. This contrasts with the situation in mammals where studies may be complicated by the continuous interaction between maternal and foetal/neonatal metabolism.

The large lipid flux through the laying hen allows for rapid diet-induced modulation of the fatty acid profile of the egg lipids. Anderson *et al* (1989) reported that the laying hen/newly hatched chick is a convenient model for the study of *n-3* fatty acid metabolism and for the development of *n-3* deficiency. This study indicated that the levels of *n-3* fatty acids in the yolk, and subsequently in the plasma, brain and retina of the newly hatched chick can be quickly and conveniently modified over more than 10-fold range by feeding appropriate diets to the laying hen. Deficiency of DHA in the brain of the chick was associated with increased levels of 22:5*n-6*. Cherian and Sim (1992, 1993) have also demonstrated that appropriate feeding of the parent bird is very

effective in modulating the amount of *n-3* fatty acids in the yolk and in the embryonic tissues. In addition, it may be envisaged that the amount of yolk lipid, and consequently the absolute amount of DHA available to the embryo, may be modulated simply by using eggs of different sizes, since the amount of yolk is proportional to egg size (Noble and Cocchi, 1990).

The particular features of lipid metabolism exhibited by the chick embryo may also help to provide insights into the characteristics of DHA transport; for example, the preferential uptake of yolk phospholipid species enriched in DHA and the distribution of considerable amounts of this fatty acid into tissue triacylglycerols (Noble and Cocchi, 1990; Speake *et al*, 1993). Also, the fact that *de novo* synthesis of fatty acids from carbohydrate does not occur to any extent in the tissues of the embryo facilitates the interpretation of studies on the mass transfer of fatty acids from the yolk to the embryo (Goodridge, 1973).

An additional advantage of the avian system, as pointed out by Anderson *et al* (1989), is that in contrast to mammals where several generation of nutritional restriction are needed to produce major changes in brain DHA content, alterations in the levels of *n-3* fatty acids in the yolk lipids dramatically affect the amount of DHA in the chick brain within a single generation, enabling the possibility to study the behavioural and other consequence of *n-3* deprivation. Recent studies using this avian system have also identified a critical period in the development of the chicken when the composition of brain phospholipids is responsive to changes in fatty acid supply, and it was demonstrated that the plasticity of the embryonic and neonatal chick brain to the provision of DHA does not extent beyond 3 weeks after hatching (Anderson *et al*, 1992; Anderson, 1994).

As a final consideration, the variability in yolk fatty acid composition between different avian (and also reptilian) species (Noble, 1991) may provide an additional means of investigating the relationship between DHA supply and the brain fatty acid profile.

MATERIALS AND METHODS

2.1.ANALYSIS OF THE LIPID COMPOSITION OF YOLK, WHOLE EMBRYOS, PLASMA AND TISSUES DURING DEVELOPMENT

2.1.1.Embryos

Fertile eggs from Ross 1 broiler-breeder parents (*Gallus gallus*), fed on a standard commercial diet were obtained from a commercial poultry supplier (Ross Poultry Farm Ltd, Inverurie, Scotland) and incubated at 37.8°C and 60% relative humidity in a force draught incubator (Brinsea Products, Banwell, England) with automatic egg turning. Hatching occurred after 21 days of incubation and the chicks were maintained for either 1 day with access to drinking water but no food provision, or for 4 days with a commercial broiler starter diet provided from the second day.

2.1.2.Sample collections

At each developmental stage studied, 4 embryos were excised from the eggs and carefully separated from their associated yolk complexes (i.e. yolk plus yolk sac membrane). The intact embryos and yolk complexes were stored at - 20°C for up to 3 weeks to await lipid analysis. The data from the analyses on these samples were used to evaluate the transfer of total lipid and of specific lipid components from the yolk to the embryo. An additional group of embryos at each stage were dissected to provide samples of specific embryonic tissues - i.e. brain, liver, heart and adipose tissue. Lipid analyses were performed on 4 replicate samples of these tissues at each stage; in order to provide sufficient material for analysis, tissue samples from up to 10 embryos depending on the developmental stage were pooled to form each replicate sample. Thus tissues from up to 40 embryos were used at each time point. In the case of adipose tissue, the subcutaneous fat pads from the upper thigh area and along the sides of the abdomen and thorax (lateral thoracic and lateral ventral regions) was quantitatively dissected. The combined tissue from these sites from both sides of a single embryo is hereafter referred to as a "depot". Other, more diffuse sites of adipose tissue, such as those in the neck region and the ventromedial area, were not included in this study. Blood samples during embryogenesis were obtained from the major blood vessel of the chorioallantoic membrane which lies just beneath the shell. Blood samples from the post-hatch chick were obtained after decapitation. At each stage, 4 replicate blood samples were collected, with each replicate sample comprising blood from up to 10 embryos / chicks. EDTA was used as the anticoagulant, and the plasma was obtained by centrifugation at 3000 r.p.m. for 20 min. The samples of brain, liver, heart, adipose tissue and plasma were stored at -20°C for up to 3 weeks to await lipid analysis. The

data from these analyses were used to evaluate the distribution of lipid moieties between different embryonic tissues.

2.1.3.Lipid extraction

The lipid extraction method followed was that described by Folch *et al* (1957). Samples were minced with scissors and then homogenised in chloroform/methanol (2:1, v/v). The homogenates were then filtered through a Whatman filter paper (reference n°1, Whatman Labscales Ltd, Kent, UK) into measuring cylinders. The deposits on the filter papers were washed with an excess of the solvent.

Such chloroform/methanol extracts are known to contain sugars, urea, salts and amino acids in addition to lipids (Christie, 1982). In order to remove these non-lipid compounds an addition of a KCl solution (0.88%, w/v) was made in a 1:4 (v/v) proportion to the chloroform/methanol extracts. After shaking, the mixture was left to stand for at least 12 hours; two phases were clearly visible : the upper "aqueous phase" was discarded and the lower one containing the lipids was evaporated in a round bottom flask on a rotary evaporator (in a water bath at 65°C).

The deposit of total lipids was then dissolved in a known volume of chloroform (10 ml); an addition of few milliliters of methanol was sometimes needed if a small amount of water was present in the samples. In such cases samples were dried again, and the operation was repeated until the lipid extract in chloroform was perfectly clear. A portion (usually half) of the chloroform extract was dried in weighed round bottom flasks on a rotary evaporator as mentioned above. By weighing the round bottom flasks plus the lipid deposit, the amount of total lipid in the sample was calculated. Portions of the remainder of the lipid extract (i.e. that which was not dried for gravimetric determination) were used for the determination of the fatty acid composition of the total lipid and of the individual lipid classes as isolated by thin layer chromatography.

2.1.4.Separation of lipid classes by thin layer chromatography

20 x 20 cm glass plates were cleaned with cotton wool dampened with hexane. The stationary phase was Kieselgel 60 G (Merck, ATG, Darmstadt, Germany). This phase was applied to the plates using an aluminium spreader which was adjusted to deliver a layer of silica gel of defined thickness (0.25 mm). Plates were allowed to dry for 30 min at room temperature and were then activated in an oven for a minimum period of half an hour at 110°C. Plates were left to cool at room temperature before sample application.

The eluting solvent was hexane/diethyl ether/formic acid (80:20:1, v/v/v), prepared freshly before use. 200 ml of the eluting solvent was added to the chromatank, which was lined with filter paper and sealed with a glass cover, and the system was allowed to equilibrate for 30 min before beginning the chromatography.

Lipid extracts were dried under a nitrogen stream (oxygen free). 5 mg of total lipid dissolved in chloroform was applied to the plate at 1 cm from the base, using a 500 µl glass syringe. The applications were allowed to dry at room temperature before the plates were placed carefully in the chromatank. When the solvent front reached 1 cm from the top of the plate (after approximately 45 min.) the plates were removed from the chromatank and air dried for a few seconds. They were then sprayed with 2'7' dichloro-fluorescein (0.1 %, w/v in methanol) and the bands representing the lipid classes were visualised under ultraviolet light. The following diagram shows the lipid class separation obtained under these conditions:

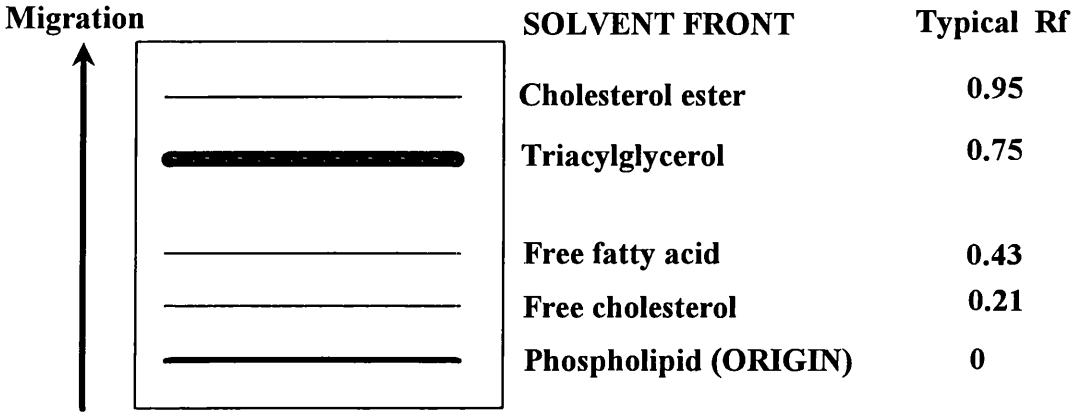


Figure 2.1: Separation of lipid classes by thin layer chromatography.

Bands were scraped from the plates with a spatula and all lipid classes were extracted by diethyl ether except for the phospholipid band where methanol was used. This operation was repeated 3 times each using 2-3 ml of solvent and the lipid extract was separated from the silica by centrifugation at 3000 r.p.m. for 3 min.

After each centrifugation, the solvent phase was poured into a round bottom flask. Each of the lipid classes were methylated as described in the following paragraph except for free cholesterol which was stored in solution in glass vial at - 20°C until analysis (see *2.1.7*).

2.1.5.Methylation of fatty acids

The methylation was carried out using 5 mg of total lipid or on lipid class extracts which had been isolated by thin layer chromatography. 1 ml of a standard

solution of 15:0 (0.322 mg/ml in methanol) was added to all extracts. A diluted solution of 15:0 (0.0322 mg/ml) was used for samples with low levels of lipid (e.g. the free fatty acid class). 15:0 was used as internal standard as it is not found naturally in animal tissues (Christie, 1982). Samples were dried in round bottom flasks on a rotary evaporator; 4 ml of methanol/toluene/sulphuric acid (20:10:1, v/v/v) was added and the samples were refluxed for 35 min at 60°C. The samples were then allowed to cool and 10 ml of water and 10 ml of hexane were added. After being shaken vigourously, 2 layers formed quickly, the top hexane layer was pipetted off and added to a test tube containing a few mg of drying reagent (sodium sulphate/sodium hydrogen carbonate; 4:1, w/w) and left for 30 minutes at least. The samples were dried down at 60°C under a flux of nitrogen (oxygen free), dissolved in few drops of hexane and injected onto the gas chromatography column.

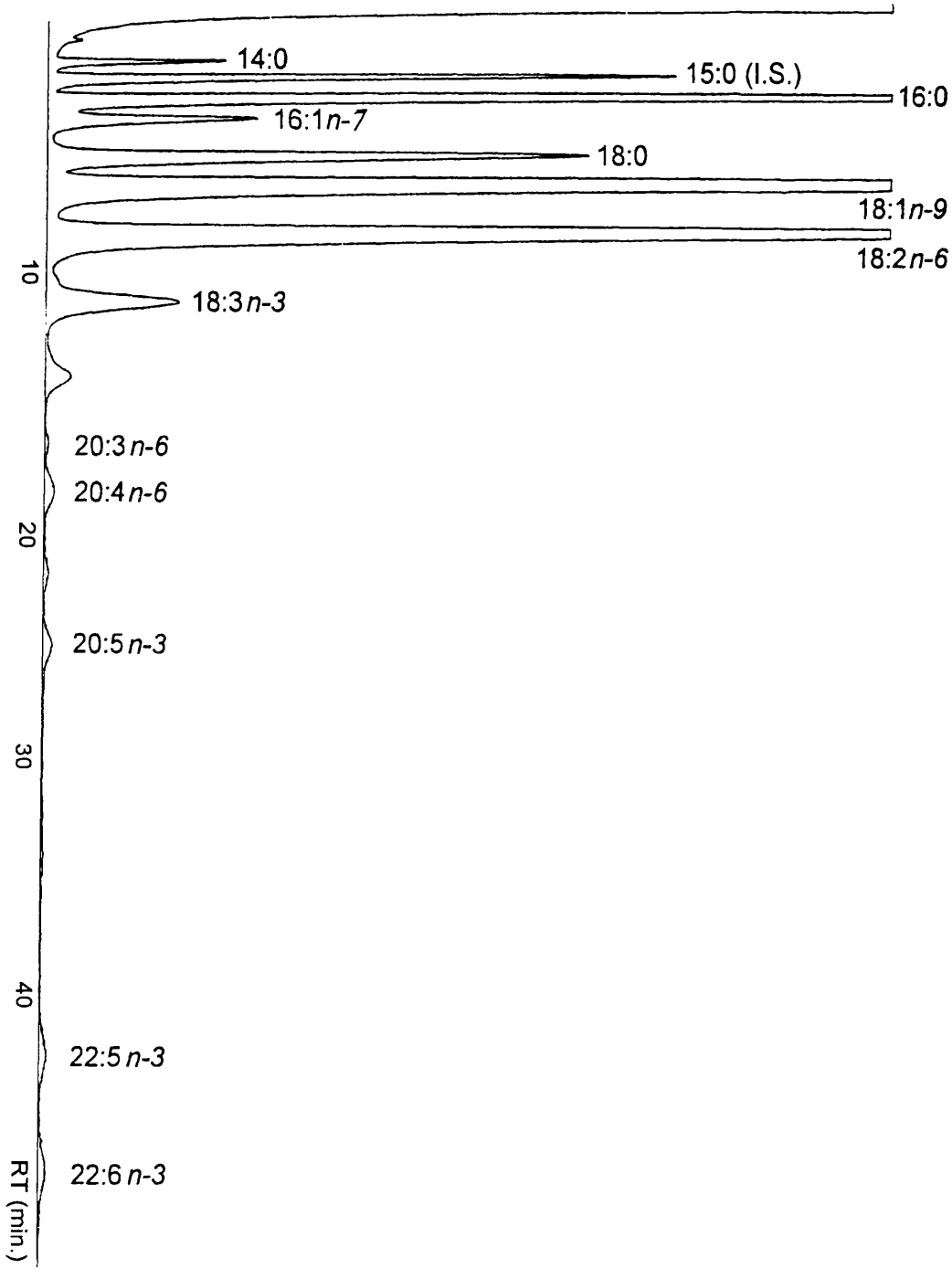
2.1.6. Determination of fatty acid composition by gas liquid chromatography

The samples were injected on an AMS 94 gas chromatograph linked to a Spectra Physics Chromjet integrator. The chromatograph was fitted with dual packed columns. The columns used were packed with 15% CP Sil 84 on Chromosorb W-HP (Chromopak UK Ltd., Middelburg, Netherlands). The characteristics were programmed as follows:

Injector temperature	220°C
Detector temperature	225°C
Oven temperature	190°C
Time of analysis	45min.
N_2 flow rate	20ml/min.
H_2 flow rate	25ml/min.
Air flow rate	250ml/min.
Amount injected	1.5 - 2µl

The retention times of the relevant fatty acid methyl esters were determined using standards. The output from the integrator displayed the shorthand names of fatty acids, their retention times, their concentration as percentages of total fatty acids and the areas of each fatty acid peak. The total amount of fatty acid methyl ester (µg) in the sample was also given by the integrator. From this value, the amount of each lipid class in a given sample was calculated after multiplication by a correction factor relating the molecular mass of the lipid class to that of the constituent fatty acid (Christie *et al.*, 1970). Figure 2.2 shows a chromatogram with peak identification.

Figure 2.2: Typical chromatogram showing fatty acid methyl esters and their retention times, RT : retention time; I.S. : internal standard.


14:0
15:0 (I.S.)
16:0
16:1n-7
18:0
18:1n-9
18:2n-6
18:3n-3
20:3n-6
20:4n-6
20:5n-3
22:5n-3
22:6n-3
10
20
30
40
RT (min.)

2.1.7. Determination of free cholesterol

Boehringer commercial test kits (Cat No 139050) were used for free cholesterol determination (Lewes, East Sussex, England). A microplate method was devised and is described below.

A colorimetric method was used. Cholesterol is oxidized by cholesterol oxidase to cholestenone (1). In the presence of catalase, the hydrogen peroxide produced in this reaction oxidizes methanol to formaldehyde (2). The latter reacts with acetylacetone forming a yellow lutidine-dye in the presence of $NH4^+$ ions (3).

(1) Cholesterol + O_2 $\xrightarrow{\text{cholesterol oxidase}}$ Δ^4 – cholestenone + H_2O_2

(2) Methanol + H_2O_2 $\xrightarrow{\text{catalase}}$ formaldehyde + 2 H_2O

(3) Formaldehyde + NH_4^+ + 2 acetylacetone $\longrightarrow$ lutidine - dye + 3 H_2O

The concentration of the lutidine-dye (3,5 diacetyl-1,4-dihydrolutidine) formed is stoichiometric with the amount of cholesterol and is measured by the increase of absorbance in the visible range at 405 nm.

40 samples were analysed per microplate. Free cholesterol extracts were dried down at 60°C under a nitrogen flux. A precise volume of propan-2-ol (IPA) was added as a function of expected cholesterol concentration (from 2 ml for the highest to 0.5 ml for the lowest concentration). Standard solutions of cholesterol in IPA were prepared, ranging in concentration from 0.1 to 1 mg/ml. 20 µl aliquots of standards and samples were placed into the microplate wells using a Gilson P20 pipette. The color reagent was added using an 8 way pipettor which delivered a volume of 250 µl into each well. The microplate was shaken for 15 sec. using the plate reader (Dynatech MR 5000 linked to a star LC-10 printer). Incubation time and temperature were as recommended by the manufacturer (60 - 75 min at 37 - 40°C). The optical densities (λ = 405 nm) were measured using the plate reader and the sample concentrations were calculated using a linear regression obtained from the standard solutions. All standards and samples were measured in duplicate.

2.2.ANALYSIS OF PHOSPHOLIPID CLASSES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY

2.2.1.Sample preparation

The total lipid extract of brain, mainly composed of phospholipid, was obtained as described above. The extract of phospholipids was evaporated under a nitrogen stream (oxygen free) in a dry block at 60°C. An appropriate amount of hexane was added to obtain a concentration of 40 mg/ml.

2.2.2.High performance liquid chromatography.

The system consisted of an SP 8800 ternary High Performance Liquid Chromatography pump (Spectra Physics Analytical Ltd., San Jose, California), an evaporative light scattering detector (Varex, Maryland, USA) and a Shimadzu C-R6A Chromatopac integrator (Dyson Instruments Ltd., Houghton-le-Spring, UK). A 10cm x 5mm column packed with Spherisorb™ 3 μ Silica (Phase Separations Ltd., Clwyd, UK) was used. The mobile phase comprised a ternary solvent system of gradually increasing polarity:

Solvent A : hexane : tetrahydrofuran 99:1 (v/v)
Solvent B : isopropanol : chloroform 4:1 (v/v)
Solvent C : isopropanol : water 1:1 (v/v)

Solvent were degassed with helium. The conditions were programmed as follows :

Time (min)	% Solvents		
	A	B	C
0	100	0	0
5	80	20	0
5.1	42	52	6
25	32	52	16
25.1	30	70	0
30	100	0	0
35	100	0	0

Maximum pressure 4500 psi
Minimum pressure 1 psi
Solvent flow rate 2 ml/min

The use of a stream splitter made the collection of the separated phospholipid species possible (4 fractions, Figure 3.20), splitting the flow by a ratio of approximately 1:10 (detected:collected). After adding 100 µl of pentadecaenoic acid standard (0.322 mg/ml) using a positive displacement pipette, the fractions were dried down in a dry block under nitrogen at 60°C and 0.5 ml of methylating reagent was added (methanol/toluene/sulphuric acid 20:10:1). Following the reflux for 30 min in the dry block at 60°C and then cooling, the methyl esters were extracted by addition of distilled water:hexane 1.5:1 (v/v) and then shaken. The upper layer (organic solvent) was removed using a Pasteur pipette and evaporated as described above. The lipid deposit was redissolved with a suitable volume of hexane (approximately 20 µl) in order to be injected on a gas chromatograph.

2.2.3. Gas liquid chromatography

The samples were analysed in the gas chromatography system described in *2.1.6.* The amounts of each phospholipid class were subsequently obtained from the gas chromatograph data since the light scattering mass detector can only be used for quantitation after extensive calibration. Figure 3.20 shows a chromatograph of the phospholipid species separation on HPLC. Peaks were identified by injections of standards onto the HPLC system: phosphatidyl-ethanolamine, phosphatidyl-serine, phosphatidyl-inositol, phosphatidyl-choline (Sigma, Dorset, England). These identifications were confirmed by analysis of the peaks isolated by HPLC on High Performance Thin Layer Chromatography (HPTLC) plates.

2.2.4. Identification of phospholipid classes by HPTLC

Precoated HPTLC silica gel 60 plates (10x10 cm) were obtained from Whatman International Ltd, Maidstone, England. Phospholipid extracts and the phospholipid classes isolated by HPLC were dried down under nitrogen and redissolved in 80 µl of chloroform:methanol (2:1, v/v). The HPTLC plates were predeveloped fully in hexane:diethyl ether (1:1, v/v) to remove impurities. After drying at room temperature, the top 1 cm was scraped off. The plate was activated at 110°C for 1 hour. 1 µl of lipid sample was applied as a spot at 1 cm from the bottom of the plate. The plates were run at room temperature in saturated tanks designed for 10x10 cm HPTLC plates. The solvent used for the separation of phospholipid classes was methyl acetate : isopropanol : chloroform : methanol : 0.25% KCl (25:25:25:10:9 by volume). After evaporation of the solvent, the plates were dried in a vacuum dessicator for 30 min. Separated phospholipid classes were detected by spraying the plate with 3% cupric acid (w/v) in

8% phosphoric acid (v/v), followed by charring at 160°C for 15 min (Olsen *et al*, 1989). Figure 2.3 shows an example of the separation with identification of the various components according to Olsen and Henderson, 1989. The individual phospholipid classes chromatographed on the plate were those isolated by HPLC.

2.3.COMPARATIVE MOBILISATION OF DIFFERENT FATTY ACIDS FROM ADIPOSE TRIACYGLYCEROL DURING INCUBATION OF ADIPOSE TISSUE *IN VITRO*

2.3.1. Incubation of adipose tissue

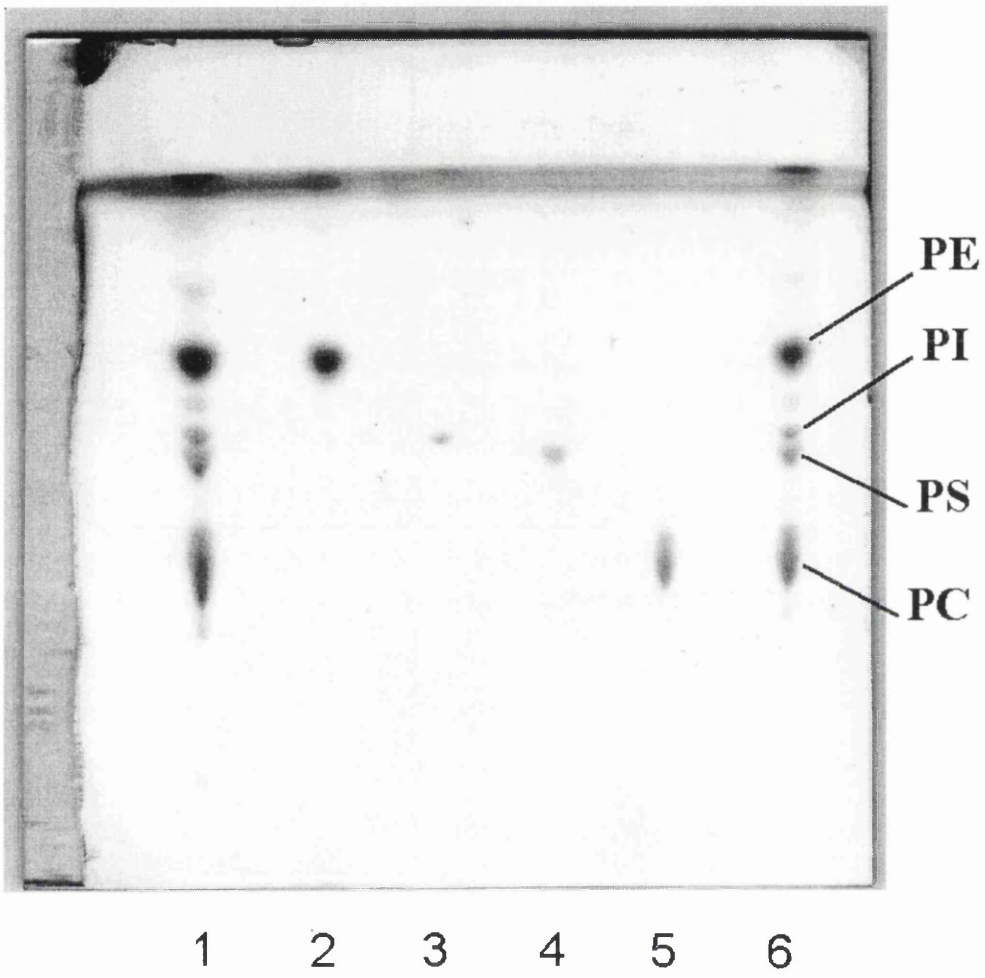
Samples of adipose tissue were obtained from embryos and chicks at appropriate stages of development as described in *2.1.* The freshly dissected tissue portions were initially collected into Dulbecco's Modified Eagle's Medium (Hepes buffered) supplemented with 1% (v/v) of antibiotic-antimycotic solution (penicillin / streptomycin / amphotericin) and 2% (w/v) of bovine serum albumin (BSA, fatty acid free) maintained at 30°C. The tissue portions were chopped into small pieces (approximately 2 mm^3) and for each replicate incubation approximately 300 mg of the tissue pieces were incubated in 1 ml of collection medium for 1 h at 37°C in a stoppered test tube with gentle shaking. Initially, polypropylene test tubes were used but because of problems caused by plasticisers interfering with the lipid analyses, these were replaced by glass tubes. Glucagon (porcine) was, where indicated, included in the incubation medium at final concentrations of 100 ng/ml or 1 μg/ml.

All components of the incubation media were obtained from Sigma Chemical Co. (Poole, Dorset, UK), except the serum albumen which was obtained from Advanced Protein Products Ltd (Bromsgrove, UK).

2.3.2. Fatty acid analysis

The aim was to compare the fatty acid composition of the mobilised free fatty acids released into the medium with that of the tissue triacylglycerol from which they were derived. Portions of unincubated tissue and of tissue and medium after incubation were extracted with a suitable excess of chloroform / methanol / HCl (10:5:1, v/v/v) following the method described in *2.1.3.* Triacylglycerol and free fatty acid fractions were isolated by thin layer chromatography (section *2.1.4.*), and their fatty acid compositions were determined by gas chromatography (section *2.1.6.*).

Figure 2.3: Separation by HPTLC of phospholipid classes. PE : phosphatidylethanolamine, PI : phosphatidylinositol, PS : phosphatidylserine and PC : phosphatidylcholine. 1 and 6 : total phospholipid extract, 2 : PE, 3 : PI, 4 : PS and 5 : PC.


PE
PI
PS
PC
1
2
3
4
5
6

2.4.STUDIES ON EMBRYOS OF OTHER AVIAN SPECIES

During the course of this study, a single experiment was carried out using embryos of the duck (*Anas platyrhynchos*). Fertile duck eggs were obtained from a commercial producer. The incubation conditions were as described for the chicken except that a higher degree of humidity was maintained by spraying the eggs twice a day with water using an aerosol can. The incubation time of duck eggs is 28 days. Samples of duck embryo brain were obtained at appropriate developmental stages. Also a single experiment was performed using eggs of the wild pheasant (*Phasianus colchicus*). The parent birds were free-living in a shooting estate in Galloway Scotland, laying the eggs in ground nests. The eggs were collected within 24 hours of laying with landowner's permission. The eggs were incubated at 37.5°C and 60% relative humidity. The incubation period for pheasant eggs is 24-26 days, and samples of adipose tissue were obtained at 16 days.

2.5.STATISTICAL ANALYSIS

Where indicated, statistical comparisons were performed using a 2 tail Student's t test or analysis of variance.

RESULTS

3.1.TRANSFER OF DHA FROM THE YOLK TO THE TISSUES OF THE EMBRYO

In this study, the changes during development in the amount of DHA in the lipids of the yolk, whole embryo, plasma, adipose tissue, liver, heart, and brain were quantified. The intention was to evaluate the rate of transfer of this fatty acid from the yolk and its subsquent distribution into specific tissue lipids, with the aim of obtaining information regarding the mechanism and route by which DHA is delivered to the brain.

3.1.1.Characteristics of eggs prior to incubation.

In order to study the effect of variations in the amount of lipid available to the embryo, and in particular the consequences of differences in the supply of DHA from the yolk, 2 experiments were performed using batches of eggs which differed in initial weight and therefore in initial yolk lipid content. These batches are henceforth referred to as "small" (experiment 1) and "normal" size (experiment 2) eggs and were obtained from laying birds of 23 and 33 weeks of age respectively.

The results in Table 3.1 indicate that the initial amount of total lipid was 1.5-fold greater in the "normal" compared to the "small" eggs. The results in Tables 3.2 and 3.3 indicate that the percentage composition of fatty acids in triacylglycerol and phospholipid were very similar in the 2 experiments, and that most of the initial yolk DHA was present as a component of phospholipid.

3.1.2.Changes in lipid composition and DHA content of the yolk complex during development.

The changes in the total lipid content of the yolk complex (i.e. yolk plus yolk sac membrane) during development are depicted in Figure 3.1. In both experiments, there was very little removal of yolk lipid during the initial period of incubation, followed by a very rapid mobilisation during the final week, in agreement with previous work (see section *1.3.2*). In the "small" egg experiment, the proportion of cholesteryl ester in the total lipid increased dramatically by the end of the embryonic period with corresponding decreases in the proportion of triacylglycerol and phospholipid (Table 3.4). However, development within the "normal" size eggs was characterised by a relative constancy in the proportions of the lipid classes (Table 3.5, Figure 3.2). The percentage fatty acid composition of the total lipid of the yolk complex showed very little change throughout development in both experiments. For example, the proportionate levels of the major fatty acids present, i.e. 16:0, 18:0, 18:1, 18:2, at day 22 (i.e. one day post hatch) were

not significantly different from the initial yolk values. In contrast, the proportion of DHA was significantly reduced during development in both experiment, by 66 % and 53% in the "small" and "normal" size eggs respectively (Tables 3.6 and 3.7). This observation suggests that DHA may be transferred preferentially from the lipids of the yolk. The percentage fatty acid compositions of the TG, PL and CE of the yolk complexes throughout development for the "normal" size egg incubation are shown in Tables 3.8, 3.9 and 3.10 respectivelly. There were no significant changes in the fatty acid composition of the TG fraction during development. However, the fatty acid composition of the PL fraction did show some changes; in particular the proportion of DHA decreased some 8-fold between day 12 and 22, consistent with previous evidence that PL-species enriched in DHA are preferentially taken up from the yolk (section *1.3*). The fatty acid composition of the CE fraction changed dramatically during development; the proportion of 18:1 increased significantly with corresponding decreases in the proportions of 16:0 and 18:2. These changes are consistent with the specific synthesis of cholesteryl oleate by the ACAT reaction in the yolk sac membrane, as previously documented (section *1.3.2*).

The amount (mg/yolk) of the major fatty acyl components of the total lipid of the yolk complex during development are given in Tables 3.11 and 3.12. In order to express these changes on the same scale, the amounts of each fatty acid are depicted as a % of the respective amount in the initial yolk (Figures 3.3 a and b). For purposes of clarity, only the values for 16:0, 18:1 and DHA are shown. The results in Figure 3.3 indicate that the 2 most abundant fatty acyl components of yolk lipid, 18:1 and 16:0, exhibited identical transfer kinetics. In addition (data not shown), the transfer of 18:0, 18:2, 18:3 and 20:4 followed a pattern identical to that of 18:1 and 16:0. In contrast, during the final week of embryogenesis, DHA in both experiments was transferred from the yolk complex at a significantly higher rate than exhibited by the other fatty acids.

3.1.3. Changes in the lipid composition and DHA content of the whole embryo during development

The major parameters relating to the lipid composition of the whole embryo during development are presented in Tables 3.13 and 3.14 and the changes in the amount of total lipid per embryo are depicted in Figure 3.4. It can be seen from these results that the total lipid content per embryo was 1.5-fold greater by the time of hatching when development occurred in the "normal" size eggs compared to the "small" eggs; i.e. closely reflecting the differences in the initial amounts of yolk lipids.

The changes in the proportions of TG and PL in the whole embryos are illustrated in Figure 3.5. During the first half of development, PL was by far the major lipid class present in the embryo. However during the final week of development the proportion of PL declined in parallel with a dramatic increase in the proportion of TG

with the "crossover point" occurring around day 14. The amounts (mg/embryo) of the different lipid classes in the whole embryo during development are depicted in Figure 3.6. The final week of development was characterised by a dramatic rise in the amount of TG in the embryo, co-incident with the phase of rapid lipid transfer from the yolk, followed by a decrease in the TG content over the hatching period. The amount of TG deposited during development reflected the amount present in the initial yolk, whereas the amounts of PL, CHOL and CE in the newly-hatched chick were less affected by the initial yolk lipid content (Figure 3.6 a and b). The fatty acid composition (% w/w of total fatty acids) of the major lipid classes are presented in Tables 3.15, 3.16, 3.17, 3.18, 3.19 and 3.20. A consistent pattern was observed in which the proportions of DHA and also AA, in TG and CE were relatively high at around day 12 and then decreased as development proceeded towards hatching. The proportion of DHA in PL was also high at around day 12 but decreased only slightly thereafter. In experiment 1 where measurements were taken as early as day 6, it was noticeable that the proportion of DHA in all 3 lipid classes was very high even at this early stage.

In order to express the changes in the levels (mg/embryo) of each fatty acid on the same scale, and to facilitate comparisons with the changes in the yolk, the values are depicted in Figure 3.7 in terms of the amount of the fatty acid per embryo as a percentage of the amount present in the lipids of the initial yolk. For clarity, only the results for DHA, AA and 18:1 are shown. It is clear from Figure 3.7 that the rate of DHA and AA accumulation in the embryo lipids during the early part of the final week of embryogenesis was especially high in comparison to 18:1. Furthermore (data not shown), the accumulation of 16:0, 18:0, 18:2 and 18:3 followed a pattern essentially identical to that of 18:1.

The results in Figure 3.7 also indicate that the amount of DHA which accumulated in the embryo, expressed as a percentage of the initial yolk level of this fatty acid, was considerably less during incubation of the "normal" size eggs in comparison to the "small" eggs. This suggests that a greater proportion of the transferred DHA was oxidised for energy provision during incubation of the "normal" size eggs. Table 3.21 depicts the levels of the major fatty acids recovered in the lipids of the day-old as percentages of the amounts transferred from the yolk by this stage. This results suggest that AA and DHA are relatively resistant to β-oxidation in the embryo in comparison to the C16 and C18 fatty acids, and that these differences on oxidative susceptibility were more pronounced during development within the "small" eggs.

Comparison of the levels of TG and PL recovered in the day-old chick with the amounts transferred from the yolk indicates that major proportions of both lipid classes were utilised for energy purposes during development (Table 3.22). These results also suggest that a greater proportion of the transferred TG was oxidised during incubation of the "small" eggs, whereas the reverse was the case for PL.

The distributions of DHA and 18:1 between TG and PL of the whole embryo for experiment 2 are illustrated in Figure 3.8. The amount of 18:1 (mg/embryo) in the TG fraction increased continuously until hatching, whereas much lower levels were present in PL. In contrast, the incorporation of DHA into TG followed a different pattern, reaching a plateau level by day 15 which was maintained until day 19, then decreasing dramatically over the hatching period. The incorporation of DHA into PL increased continuously from day 13 so that by day 19 this fatty acid was almost equally distributed between PL and TG.

Table 3.1: Characteristics of "small" (experiment 1) and "normal".(experiment 2) size eggs prior to incubation. Values are the means ± S.E.M. of measurements on 4 eggs with the exception of egg weights where n=10. The amounts (mg/yolk) of DHA and of other major fatty acids in the total lipids of the yolk are shown. Significant differences for values on the same line (between experiments) are illustrated with superscript character $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$, $^{n}P>0.05$ not significant.

	Experiment	
	1	2
Age of parental flock (weeks)	23	33
Egg weight (g)	46.3 ± 0.4	62.2 ± 0.7 ^{c}
Total lipid (g/yolk)	3.8 ± 0.1	5.7 ± 0.1 ^{c}
Lipid class % (w/w) of total lipid		
TG	80.4 ± 6.2	70.5 ± 0.4 ^{n}
PL	13.0 ± 3.0	20.5 ± 0.8 ^{n}
CHOL	4.7 ± 1.2	6.6 ± 0.6 ^{n}
CE	2.0 ± 1.1	1.7 ± 0.1 ^{n}
FFA	3.6 ± 1.0	0.86 ± 0.07 ^{a}
mg fatty acid/yolk		
16:0	681.2 ± 34.0	1086.5 ± 27.3 ^{c}
18:0	267.1 ± 14.9	413.8 ± 12.0 ^{c}
18:1	1144.8 ± 77.6	1757.7 ± 61.6 ^{c}
18:2	469.5 ± 31.0	689.6 ± 71.1 ^{a}
18:3	30.2 ± 2.9	54.1 ± 6.2 ^{a}
20:4	61.1 ± 7.0	73.1 ± 3.8 ^{n}
22:6	32.9 ± 3.0	93.8 ± 4.7 ^{c}

Table 3.2: Fatty acid composition of yolk triacylglycerol (% w/w of total fatty acid). Details are as described in Table 3.1.

	Experiment	
	1	2
16:0	24.7 ± 0.3	25.1 ± 0.7 [n]
18:0	6.2 ± 0.2	6.9 ± 0.4 [n]
18:1	40.6 ± 0.5	45.7 ± 0.1 [c]
18:2	21.0 ± 0.4	15.3 ± 1.4 [b]
18:3	1.9 ± 0.1	1.4 ± 0.2 [n]
20:4	0.42 ± 0.03	0.27 ± 0.02 [b]
22:6	0.39 ± 0.03	0.22 ± 0.02 [b]

Table 3.3: Fatty acid composition of yolk phospholipid (% w/w of total fatty acid). Details are as described in Table 3.1.

	Experiment	
	1	2
16:0	27.3 ± 0.3	27.9 ± 0.3 [n]
18:0	17.6 ± 0.3	16.8 ± 0.4 [n]
18:1	21.3 ± 0.4	24.6 ± 0.8 [a]
18:2	16.7 ± 0.2	16.0 ± 0.5 [n]
18:3	0.33 ± 0.1	0.40 ± 0.03 [n]
20:4	6.5 ± 0.2	5.5 ± 0.1 [b]
22:6	8.1 ± 0.2	6.3 ± 0.3 [b]

Figure 3.1: Transfer of total lipid from the yolk complex during development. The values for the "small" (●) and "normal" size (○) eggs are shown. The results represent the mean ± S.E.M. of measurement on 4 yolk complexes. Hatching occurred on day 21, i.e. day 22 refers to 1 day after hatching.

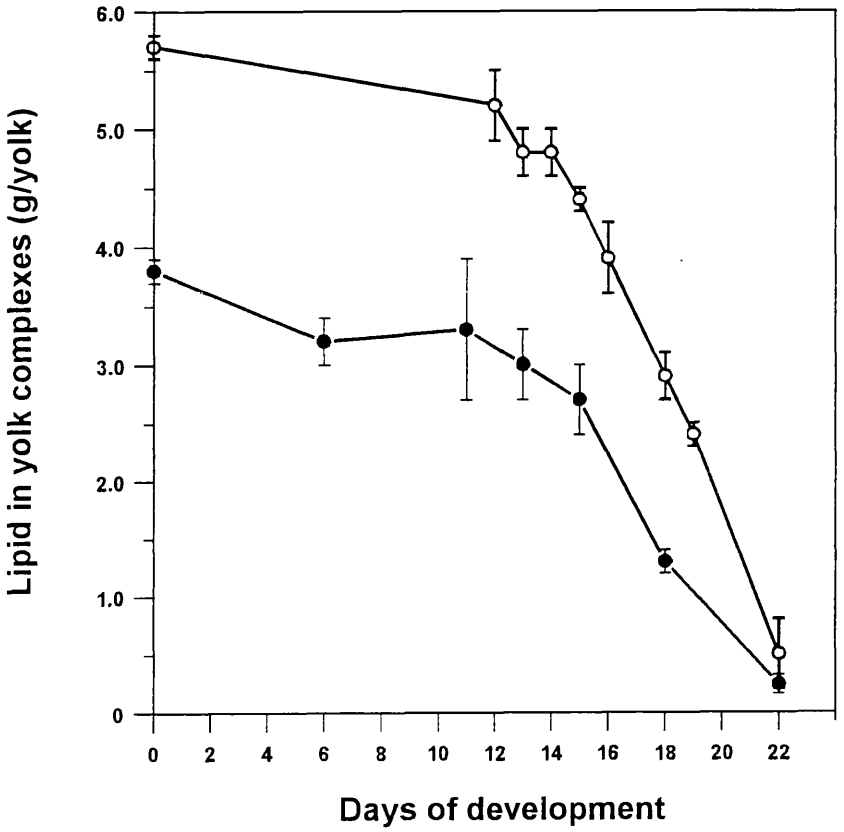

Lipid in yolk complexes (g/yolk)
6.0
5.0
4.0
3.0
2.0
1.0
0
0
2
4
6
8
10
12
14
16
18
20
22
Days of development

Table 3.4: Lipid classes of the yolk complexes from experiment 1 (% w/w of total lipid). Statistical comparison refers to day 22 vs day 18; symbols (a, b, c, n) as in Table 3.1.

	Day 6	11	13	15	18	22
TG	65.1 ± 0.7	67.9 ± 0.7	69.4 ± 3.8	64.0 ± 6.0	65.2 ±2.2 [n]	42.1 ±1.6[a]
PL	25.1 ± 1.6	19.3 ± 1.4	16.7 ± 1.0	25.0 ± 6.7	20.2 ± 1.7	14.5 ±0.3[a]
CHOL	5.3 ± 0.6	4.6 ± 0.3	4.6 ± 0.5	4.2 ± 0.4	4.1 ± 0.2	4.4 ± 0.6[n]
CE	2.6 ± 0.5	3.3 ± 1.0	3.5 ± 0.7	4.2 ± 0.4	9.1 ± 0.8	35.1 ±2.3[a]
FFA	1.9 ± 0.2	5.0 ± 2.2	6.0 ± 2.6	2.8 ± 1.7	1.4 ± 0.2	2.8 ± 1.1
Amount of total lipid (g/yolk)	3.2± 0.2	3.3± 0.6	3.0 ± 0.3	2.7 ± 0.3	1.3 ± 0.1	0.24±0.08

Table 3.5: Lipid classes of the yolk complexes from experiment 2 (% w/w of total lipid). Statistical comparison refers to day 22 vs day 18; symbols (a, b, c, n) as in Table 3.1.

	Day 12	13	14	15	18	19	22
TG	71.3 ± 2.3	70.1± 2.2	70.6± 2.0	68.7± 0.5	72.8± 0.4	72.4± 0.7	77.9 ±0.9[b]
PL	19.9 ± 1.6	20.4± 1.6	21.1 ±1.0	22.8 ±1.7	24.1 ±1.0	20.1± 0.6	13.2± 1.6[b]
CHOL	4.4 ± 0.6	3.9 ± 0.5	5.3 ± 2.4	3.6± 0.1	2.6 ± 0.7	3.9 ± 0.6	6.6 ± 1.8[n]
CE	1.6 ± 0.2	1.2 ± 0.2	2.5 ± 0.3	2.5 ± 0.7	1.7 ± 1.0	3.1± 0.4	1.8 ± 0.8[n]
FFA	1.5 ± 0.6	2.8 ± 0.1	1.5 ± 0.2	1.7 ± 0.1	0.8 ± 0.3	0.9 ± 0.4	1.7± 0.8[n]
Amount of total lipid (g/yolk)	5.2 ± 0.3	4.8 ± 0.2	4.8 ± 0.2	4.4 ± 0.1	2.9 ± 0.2	2.4 ± 0.1	0.5 ± 0.3

Figure 3.2: Distribution of lipid classes of yolk complexes. The amounts (mg/yolk) of TG (●), PL (○), CHOL (■) and CE(□) were determined from experiment 1 (graphs a and b) and experiment 2 (graphs c and d) throughout development. Results are means of 4 replicate tissue samples and in all cases the SE's were less than 10 % of the means.

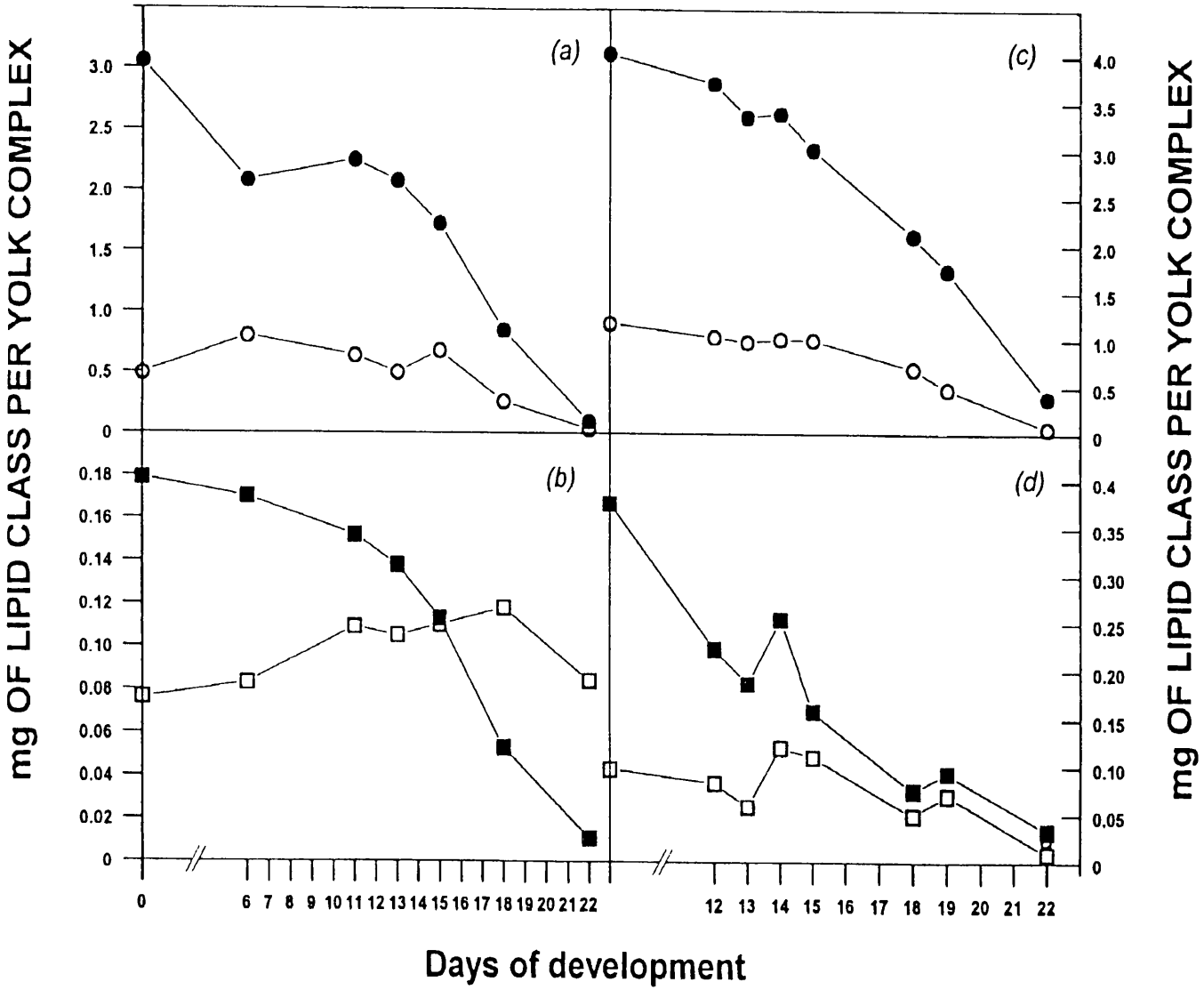

(a)
(b)
(c)
(d)
mg OF LIPID CLASS PER YOLK COMPLEX
mg OF LIPID CLASS PER YOLK COMPLEX
Days of development

Table 3.6: Fatty acid percentage composition of yolk total lipid during development (experiment 1). Results are % w/w of total fatty acids. Statistical comparison refers to day 22 vs day 0; symbols (a, b, c, n) as in Table 3.1.

	Day 0	6	11	13	15	18	22
16:0	25.4 ± 1.3	24.7 ± 1.0	24.0 ± 0.8	25.1 ± 2.3	25.0 ± 2.0	24.1 ± 2.6	20.1 ±2.5^{n}
18:0	9.9 ± 0.6	9.3 ± 0.4	8.7 ± 0.6	9.2 ± 0.6	9.1 ± 0.6	9.7 ± 1.1	9.9 ± 1.4^{n}
18:1	42.6 ± 2.9	44.3 ± 3.5	42.3 ± 1.1	41.8 ± 3.3	42.7 ± 3.3	44.0 ± 3.7	49.1 ±5.7^{n}
18:2	17.5 ± 1.1	16.8 ± 2.1	20.0 ± 2.8	18.6 ± 1.9	18.6 ± 1.1	17.6 ± 2.1	17.7 ±1.7^{n}
18:3	1.1 ± 0.1	1.1 ± 0.2	1.3 ± 0.2	1.4 ± 0.2	1.2 ± 0.1	2.1 ± 0.2	0.9 ± 0.2^{n}
20:4	2.3 ± 0.3	2.5 ± 0.2	2.3 ± 0.2	2.6 ± 0.2	2.4 ± 0.2	2.5 ± 0.3	1.9 ± 0.1^{n}
22:6	1.2 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.4 ± 0.1^{b}

Table 3.7: Fatty acid percentage composition of yolk total lipid during development (experiment 2). Results are % (w/w) of total fatty acids. Statistical analysis performed on values of 22 against values of day 0.

	Day 0	12	13	14	15	16	18	19	22
16:0	26.1 ±1.3	25.8 ±1.6	26.4 ±1.2	25.9 ±0.9	25.4 ±0.9	26.3 ±2.0	25.4 ±0.3	25.7 ±1.6	24.2± 6.5^{n}
18:0	9.9 ± 0.6	9.7 ± 0.9	10.0 ±0.4	9.4 ± 0.5	9.8 ± 0.4	9.4 ± 1.6	10.6 ±0.3	9.7 ± 1.1	10.7± 2.3^{n}
18:1	42.2 ±3.0	43.0 ±3.4	41.9 ±1.8	42.9 ±1.3	42.5 ±2.1	44.0 ±5.0	42.3 ±2.9	43.3 ±3.6	45.4±13.0^{n}
18:2	16.5 ±3.4	16.4 ±0.8	16.7 ±1.2	16.7 ±0.5	17.6 ±1.4	16.0 ±1.5	17.3 ±0.8	17.3 ±1.8	16.2± 1.7^{n}
18:3	1.3 ± 0.3	1.4 ±0.04	1.38±0.06	1.35±0.06	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	1.2 ±0.1^{n}
20:4	1.8 ± 0.2	1.7 ± 0.1	1.8 ± 0.2	1.8 ± 0.1	1.7 ± 0.1	1.6 ± 0.3	1.9 ± 0.2	1.6 ± 0.2	1.2 ±0.1^{a}
22:6	2.3 ± 0.2	2.0 ± 0.1	1.8 ± 0.2	2.0 ± 0.2	1.8 ± 0.2	1.4 ± 0.2	1.2 ± 0.1	1.1 ± 0.1	1.1 ±0.0^{b}

Table 3.8: Fatty acid composition of triacylglycerol of the yolk complex during embryonic development for experiment 2 (% w/w of total fatty acids). Statistical comparison refers to day 22 vs day 12; symbols (a, b, c, n) as in Table 3.1.

Acid	D12	13	14	15	18	19	22
16:0	26.5 ± 0.7	26.7 ± 0.9	25.9 ± 0.6	24.9 ± 0.3	26.1 ± 0.7	27.0 ± 0.6	26.4 ±0.8^{n}
18:0	6.6 ± 0.3	6.9 ± 0.2	6.4 ± 0.3	6.7 ± 0.5	6.4 ± 0.3	6.4 ± 0.1	8.4 ± 0.9^{n}
18:1	48.1 ± 0.6	47.3 ± 1.8	46.5 ± 1.4	48.3 ± 1.0	48.0 ± 1.0	46.3 ± 1.7	46.6 ±1.4^{n}
18:2	11.8 ± 0.5	11.9 ± 0.8	13.3 ± 0.8	12.1 ± 0.8	12.3 ± 0.2	12.7 ± 1.2	12.40± 0.03^{n}
18:3	0.81 ± 0.03	0.85 ± 0.03	0.97 ± 0.05	0.88 ± 0.05	0.84 ± 0.05	1.1 ± 0.3	0.83± 0.01^{n}
20:4	0.33 ± 0.07	0.33 ± 0.02	0.35 ± 0.06	0.42 ± 0.06	0.33 ± 0.02	0.37 ± 0.05	0.42± 0.03^{n}
22:6	0.48 ± 0.16	0.58 ± 0.02	0.78 ± 0.11	0.74 ± 0.05	0.44 ± 0.08	0.61 ± 0.08	0.60± 0.06^{n}

Table 3.9: Fatty acid composition of phospholipid of yolk complex during embryonic development for experiment 2 (% w/w of total fatty acids). Statistical comparison refers to day 22 vs day 12; symbols (a, b, c, n) as in Table 3.1.

Acid	D12	13	14	15	18	19	22
16:0	29.5 ± 0.3	31.3 ± 1.0	29.4 ± 0.3	31.7 ± 0.4	29.4 ± 0.5	31.1 ± 0.8	28.3 ±0.7^{n}
18:0	18.3 ± 0.2	16.7 ± 0.8	18.1 ± 0.3	15.2 ± 0.4	17.1 ± 0.4	16.8 ± 1.0	20.1 ±1.0^{n}
18:1	25.9 ± 0.4	27.8 ± 1.1	28.1 ± 0.2	28.3 ± 1.0	30.4 ± 0.5	29.6 ± 0.9	30.9 ± 0.4^{c}
18:2	12.4 ± 0.5	13.0 ± 0.5	13.4 ± 0.6	13.3 ± 0.2	13.5 ± 0.2	13.5 ± 0.6	13.8 ± 0.3^{n}
18:3	0.24 ± 0.01	0.27 ± 0.01	0.29 ± 0.01	0.30 ± 0.05	0.33 ± 0.04	0.29 ± 0.02	0.29 ±0.06^{n}
20:4	5.4 ± 0.1	4.9 ± 0.3	ND	4.3 ± 0.1	4.5 ± 0.2	3.9 ± 0.6	4.0 ± 0.1^{c}
22:6	6.03 ± 0.11	5.0 ± 0.3	4.87 ± 0.04	4.3 ± 0.3	2.4 ± 0.3	2.20 ± 0.08	0.76±0.09^{c}

Table 3.10: Fatty acid composition (% w/w of total fatty acids) of yolk cholesteryl ester during embryonic development for experiment 2. Statistical comparison refers to day 22 vs day 12; symbols (a, b, c, n) as in Table 3.1.

	D12	13	14	15	18	19	22
16:0	16.5 ± 1.1	18.4 ± 2.0	15.9 ± 1.4	12.8 ± 1.4	13.5 ± 2.3	8.6 ± 0.9	5.5 ± 1.0^{c}
18:0	7.7 ± 0.8	7.5 ± 0.3	8.9 ± 1.3	7.6 ± 0.5	5.9 ± 0.3	5.7 ± 0.6	5.7 ± 0.5^{n}
18:1	51.5 ± 1.4	56.2 ± 2.9	52.7 ± 2.9	60.2 ± 1.9	63.1 ± 3.7	67.4 ± 1.1	73.8±1.1^{c}
18:2	15.6 ± 0.5	13.3 ± 1.7	13.5 ± 1.2	13.2 ± 1.0	7.4 ± 0.7	10.7 ± 0.7	9.3 ± 0.2^{c}
18:3	ND	0.32 ± 0.2	0.1 ± 0.1	0.4 ± 0.2	0.5 ± 0.1	0.54±0.08	0.7 ± 0.4
20:4	1.9 ± 0.9	ND	2.4 ± 0.7	1.4 ± 0.4	1.2 ± 0.7	0.9 ± 0.1	0.8±0.03^{n}
22:6	2.4 ± 0.3	ND	4.0 ± 1.0	5.2 ± 5.2	2.1 ± 0.3	2.0 ± 0.2	1.8 ± 0.1^{n}

Table 3.11: Fatty acid composition (mg/yolk) of yolk total lipid during embryonic development for experiment 1.

	Day0	6	11	13	15	18	22
16:0	681 ± 34	559 ± 22	516 ± 18	540 ± 50	451 ± 36	226 ± 24	19.9 ± 2.5
18:0	267 ± 15	211 ± 10	186 ± 12	198 ± 12	164 ± 10	91 ± 10	9.8 ± 1.4
18:1	1145 ± 78	1003 ± 80	908 ± 23	900 ± 70	770 ± 60	412 ± 35	48.6 ± 5.6
18:2	470 ± 31	381 ± 49	430 ± 60	400 ± 40	335 ± 20	165 ± 20	17.5 ± 1.7
18:3	30 ± 3	24 ± 4	29 ± 4	30 ± 4	22 ± 2	11 ± 2	0.9 ± 0.2
20:4	61 ± 7	57 ± 4	50 ± 4	57 ± 4	43 ± 3	23 ± 3	1.9 ± 0.1
22:6	33 ± 3	31 ± 3	30 ± 2	28 ± 3	18 ± 1	8 ± 1	0.4 ± 0.1

Table 3.12: Fatty acid composition of yolk (mg/yolk) during embryonic development for experiment 2.

	Day 0	12	13	14	15	18	19	22
16:0	1087 ± 27	1067 ± 52	1038 ± 56	1008 ± 19	972 ± 33	714 ± 5	624 ± 14	182.9±4.0
18:0	414 ± 12	370 ± 22	349 ± 19	336.9±3.2	319 ± 18	248 ± 12	195 ± 3	68.6 ± 2.3
18:1	1758 ± 62	1782 ± 75	1680 ±121	1673 ± 88	1625 ± 89	1260 ± 40	1008 ± 41	346.7±7.4
18:2	690 ± 71	538 ± 32	478 ± 14	529 ± 29	471 ± 18	357 ± 8	306 ± 23	100 ± 3
18:3	54 ± 6	55.4±12.7	31.2 ± 0.9	35.5 ± 1.5	31.0 ± 1.3	24.6 ± 0.4	19.8 ± 0.9	6.2 ± 0.2
20:4	73.1 ± 3.8	69.8 ± 4.1	58.8 ± 4.0	59.0 ± 3.6	51.3 ± 2.0	40.9 ± 2.8	31.9 ± 1.4	8.9 ± 0.4
22:6	93.8 ± 4.7	98.5 ± 6.2	81.8 ± 6.3	84.6 ± 8.7	68.4 ± 1.3	39.1 ± 2.6	31.7 ± 2.2	10.9 ± 1.9

Figure 3.3: Transfer of fatty acids from the lipids of yolk complex. Changes in the level of oleic acid (○), palmitic acid (●) and DHA (□) are shown during incubation of "small" (a) and "normal" (b) size eggs. Values are expressed as percentage of the amounts in the initial yolks prior to incubation and are the means of 4 replicates. Comparison between oleic acid and DHA values : $^{b}P<0.002$, $^{c}P<0.02$, $^{d}P<0.05$.

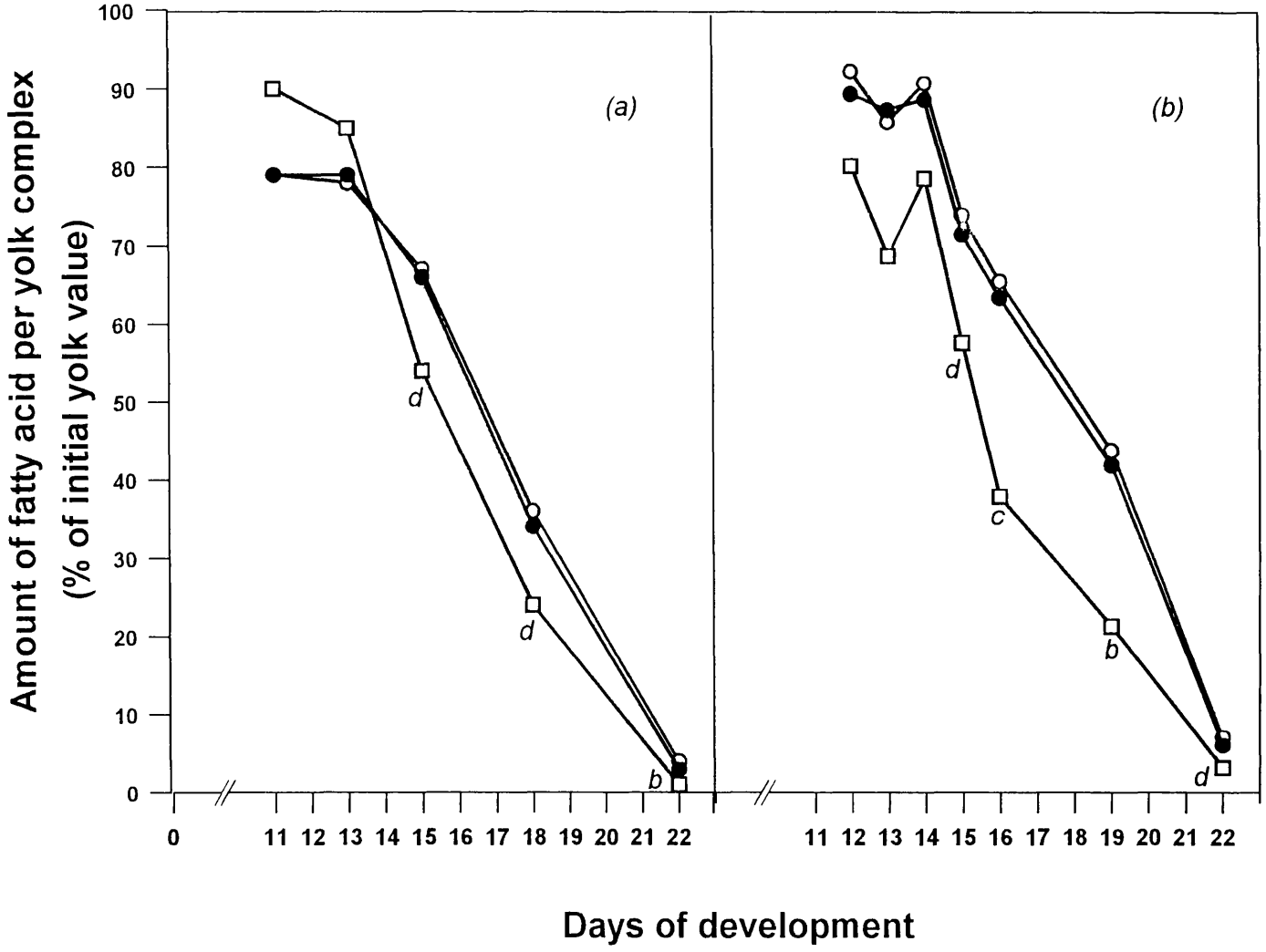

Amount of fatty acid per yolk complex
(% of initial yolk value)
100
90
80
70
60
50
40
30
20
10
0
(a)
(b)
d
d
b
d
c
b
d
0
11 12 13 14 15 16 17 18 19 20 21 22
11 12 13 14 15 16 17 18 19 20 21 22
Days of development

Table 3.13: Lipid composition and fatty acid content of whole embryos during development (experiment 1). The amount (mg) of each major fatty acid as a component of the total lipid per embryo is shown. Day 22 refers to 1 day after hatching. Results are the means ± S.E.M. of measurements on 4 embryos per time-point. Statistical analysis to the comparison between days 6 and 22 or 18 on the same line, same as Table 3.1.

	Day 6	11	13	15	18	22
Embryo wt (g)	0.36 ± 0.02	3.5 ± 0.6	8.2 ± 0.6	13.9 ± 0.4	23.8 ± 0.6	36 ± 5
Total lipid / embryo or chick (mg)	2.9 ± 0.2	45.3 ± 0.5	128 ± 15	359 ± 39	1080 ± 90	1080 ± 210
Lipid class % (w/w) of total lipid						
CE	14.1 ± 1.5	16.8 ± 2.3	14.1 ± 4.5	9.2 ± 1.9	8.9 ± 0.4	16.6 ± 4.6^{n}
TG	16.6 ± 3.4	14.2 ± 1.3	23.2 ± 3.2	45.2 ± 5.6	65.7 ± 3.5^{c}	33.7 ± 7.6^{n}
FFA	13.1 ± 0.3	4.7 ± 0.4	4.8 ± 2.5	9.1 ± 0.9	6.6 ± 0.8	17.3 ± 2.7^{n}
PL	51.9 ± 2.8	49.8 ± 7.1	42.5 ± 4.0	27.9 ± 4.6	10.2 ± 1.7	19.3 ± 2.9^{c}
CHOL	14.2 ± 4.4	14.5 ± 1.1	15.6 ± 1.9	8.6 ± 3.9	9.4 ± 1.4	13.1 ± 1.9^{n}
mg fatty acid / embryo or chick						
16:0	0.51 ± 0.03	5.30 ± 1.40	17.5 ± 2.8	60.1 ± 6.8	201 ± 19	136±32
18:0	0.24 ± 0.01	3.10 ± 0.85	9.6 ± 4.6	25.0 ± 2.2	68 ± 6	68 ± 9
18:1	0.35 ± 0.02	4.43 ± 1.41	17.8 ± 3.7	75.4 ± 11.5	269 ± 30	208 ± 42
18:2	0.09 ± 0.01	1.33 ± 0.41	8.2 ± 1.7	39.5 ± 4.6	134 ± 14	114 ± 25
18:3	0.01 ±0.001	0.04 ± 0.02	0.5 ± 0.53	2.3 ± 0.2	6 ± 1	5 ± 3
20:4	0.18 ± 0.01	3.61 ± 0.25	8.2 ± 1.4	16.4 ± 1.0	31 ± 1	44 ± 5
22:6	0.88 ± 0.01	1.78 ± 0.57	5.7 ± 0.9	12.2 ± 1.0	16 ± 1	20 ± 2

Table 3.14: Lipid composition and fatty acid content of whole embryos during development (experiment 2). The amount (mg) of each major fatty acid as a component of the total lipid per embryo is shown. Day 22 refers to 1 day after hatching. Results are the means ± S.E.M. of measurements on 4 embryos per time-point. Statistical analysis to the comparison between days 12 and 22 on the same line.

	Day12	13	14	15	16	19	22
Embryo / chick wt (g)	6.1 ± 0.9	8.9 ± 0.2	13.9 ± 1.0	16.2 ± 1.0	20.5 ± 0.6	31.9 ± 3.2	39.0 ± 2.8
Total lipid / embryo or chick (mg)	91.3 ± 2.4	110.6 ± 12.2	241.8 ± 16.1	408.5 ± 3.4	703.3 ± 65.3	1557.6 ± 235.7	1448.3 ± 156.7
Lipid class % (w/w) of total lipid							
CE	8.1 ± 1.2	7.2 ± 0.8	4.9 ± 0.2	5.7 ± 0.3	5.9 ± 0.1	6.1 ± 0.3	10.7 ± 0.4[n]
TG	18.3 ± 2.3	34.1 ± 5.4	52.7 ± 1.7	60.4 ± 4.0	66.4 ± 4.4	75.5 ± 1.5	59.3 ± 2.9[c]
FFA	3.4 ± 0.4	3.6 ± 0.8	3.3 ± 0.2	3.0 ± 0.3	2.3 ± 0.5	2.6 ± 0.5	5.4 ± 0.9[n]
PL	53.2 ± 3.5	41.8 ± 5.9	30.1 ± 0.6	25.1 ± 1.6	19.0 ± 3.3	11.8 ± 1.1	14.0 ± 2.9[c]
CHOL	16.5 ± 4.4	12.7 ± 2.8	9.1 ± 1.2	8.5 ± 0.2	6.3 ± 0.8	4.6 ± 0.3	10.7 ± 4.3[n]
mg fatty acid / embryo or chick							
16:0	11.9 ± 0.3	15.7 ± 1.9	40.0 ± 3.0	71.3 ± 0.6	108.5 ± 12.5	303.0 ± 47.1	255.3 ± 17.8
18:0	6.65 ± 0.09	8.1 ± 0.6	16.7 ± 1.3	30.8 ± 0.5	43.6 ± 4.5	94.4 ± 12.5	101.1 ± 6.2
18:1	10.2 ± 0.3	13.9 ± 1.9	42.8 ± 3.5	82.6 ± 0.5	128.3 ± 18.3	365.5 ± 47.2	365.7 ± 3.7
18:2	4.5 ± 0.5	6.0 ± 1.0	21.4 ± 2.4	44.1 ± 2.9	68.6 ± 6.2	157.9 ± 6.9	169.0 ± 20.4
18:3	0.25 ± 0.03	0.35 ± 0.07	1.5 ± 0.2	2.9 ± 0.3	4.8 ± 0.7	9.5 ± 0.7	10.7 ± 2.2
20:4	4.9 ± 0.2	5.9 ± 0.3	9.7 ± 0.4	15.0 ± 0.5	19.6 ± 1.3	21.6 ± 1.6	34.8 ± 6.2
22:6	5.71 ± 0.04	6.5 ± 0.4	12.6 ± 0.3	21.9 ± 1.9	23.2 ± 2.3	21.5 ± 2.8	28.4 ± 5.1

Figure 3.4: Changes in the amount of total lipid (mg) per embryo. The values for embryos from "small" (●) and "normal" (○) size eggs are shown. The results represent the mean of measurements on 4 embryos. Hatching occured on day 21, i.e. day 22 refers to 1 day after hatching.

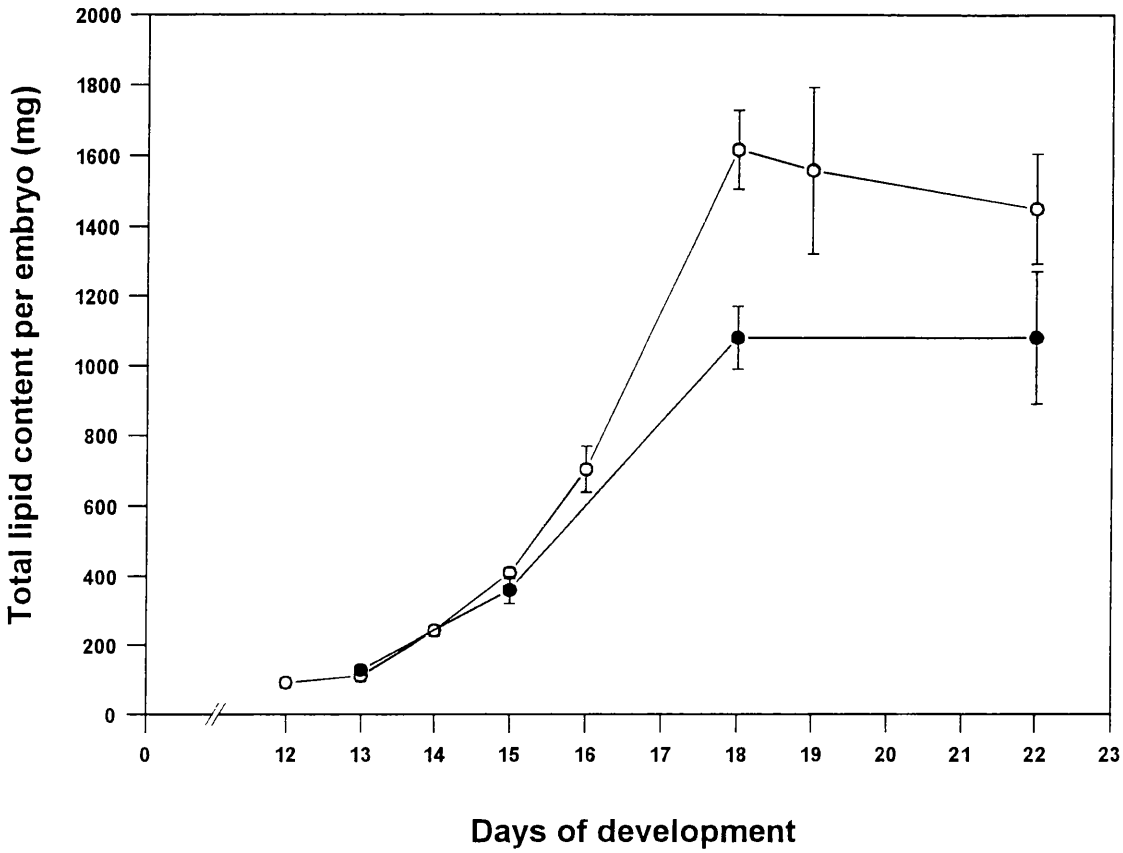

Total lipid content per embryo (mg)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
12
13
14
15
16
17
18
19
20
21
22
23
Days of development

Figure 3.5: Proportions of major lipid classes in the whole embryo throughout development. The proportions (% of total lipid) of TG (○) and PL (●) from experiment 1 (a) and experiment 2 (b) are shown. The results represent the mean of measurements on 4 embryos and are based on the data in Tables 3.13 and 3.14. Hatching occurred on day 21, i.e. day 22 refers to 1 day after hatching.

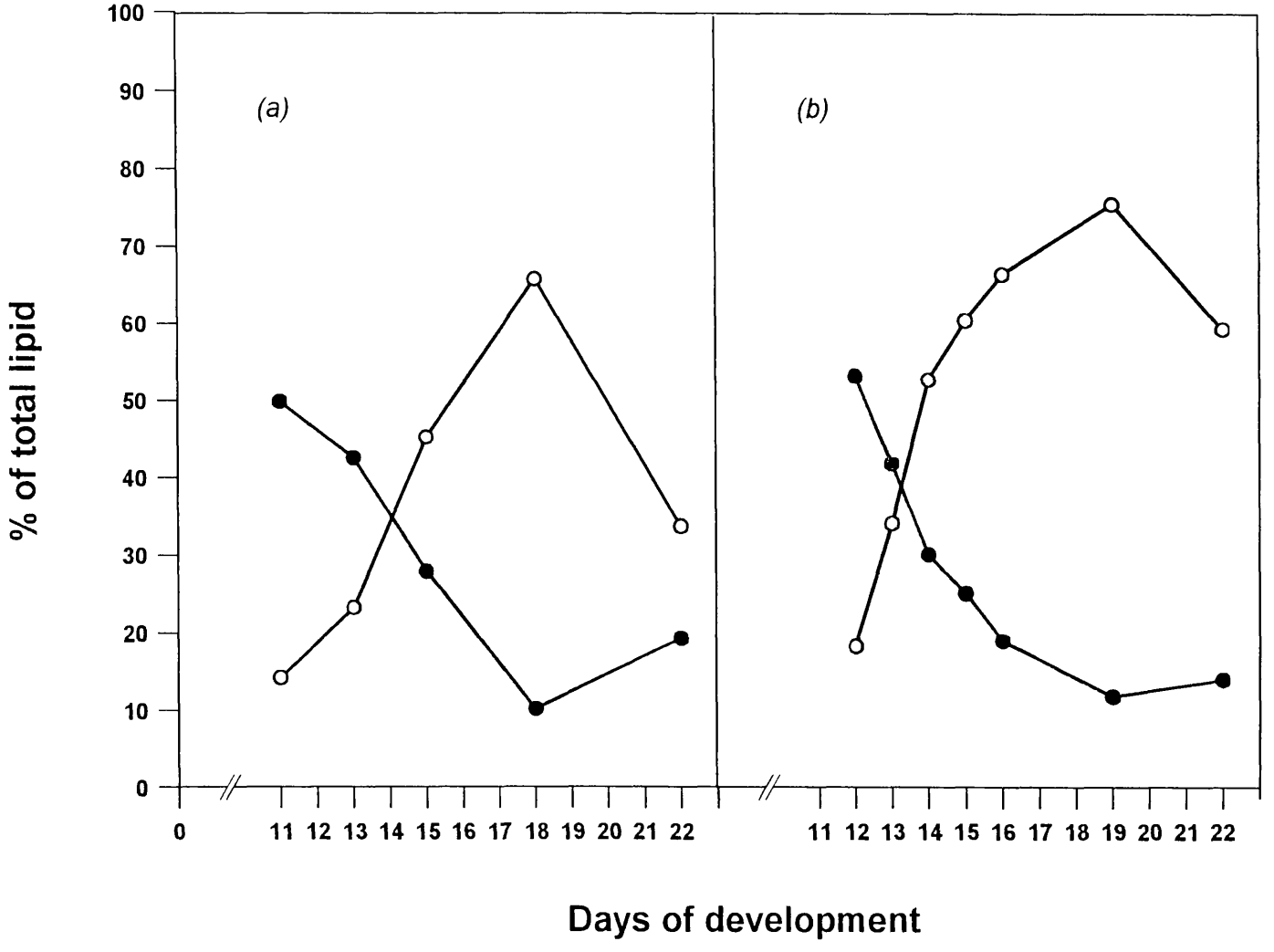

100
90
80
70
60
50
40
30
20
10
0
(a)
(b)
% of total lipid
0
11 12 13 14 15 16 17 18 19 20 21 22
11 12 13 14 15 16 17 18 19 20 21 22
Days of development

Figure 3.6: Levels of the major lipid classes in the embryo during development. The amounts (mg/embryo) of TG (●), PL (○), CE (□) and CHOL (■) are shown during incubation of "small" (a) and "normal" (b) eggs. Values are the mean of measurements on 4 embryos and are based on the data in Tables 3.13 and 3.14.

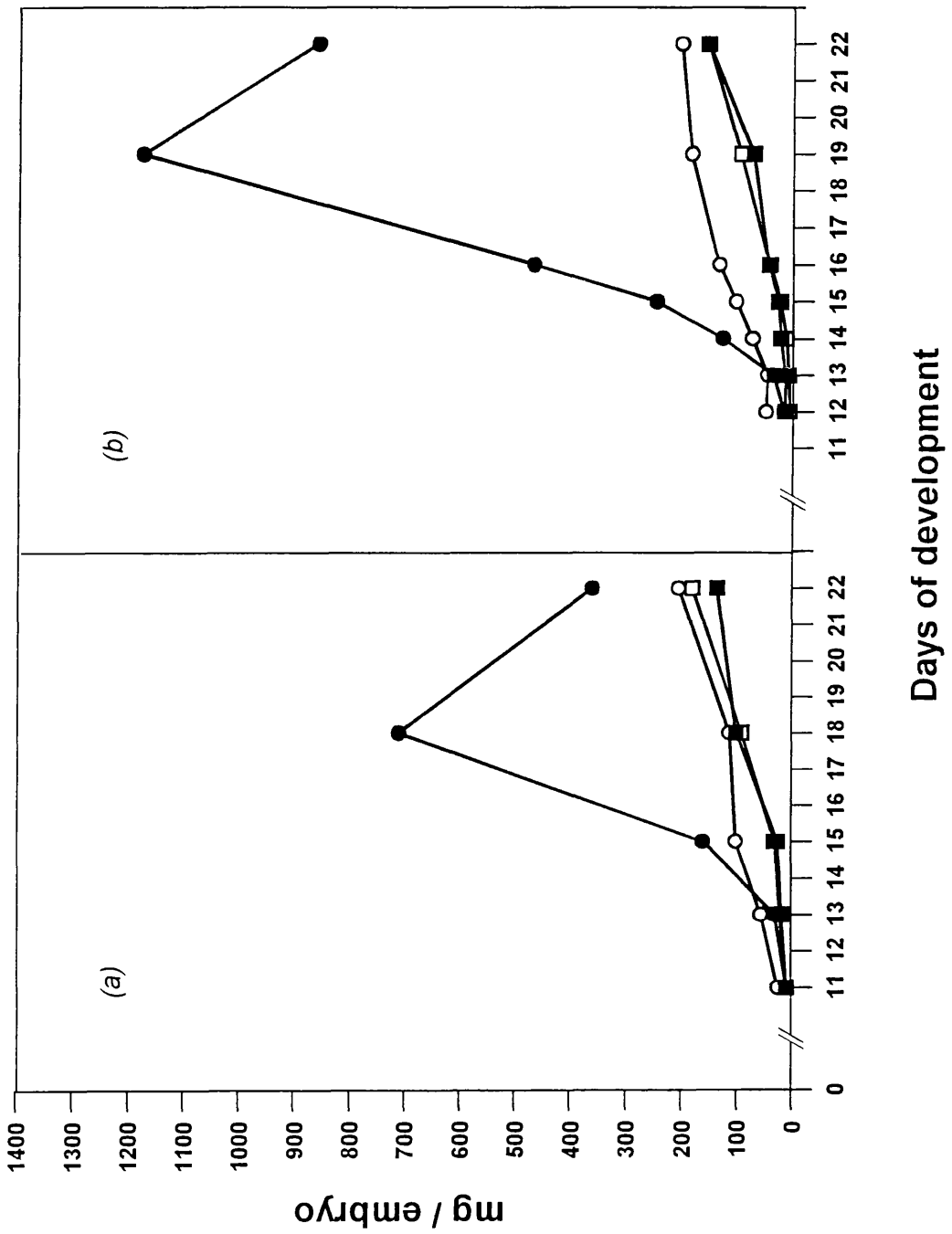

mg / embryo
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
(a)
(b)
0
11 12 13 14 15 16 17 18 19 20 21 22
Days of development

Table 3.15: Fatty acid composition (% w/w of total fatty acid; means ± S.E.M. of 4 embryos) of whole embryo triacylglycerol from experiment 1.

	Day 6	11	13	15	18	22
16:0	24.7±1.0	24.2±0.9	25.6±0.6	27.4±1.0	26.2±0.9	28.1±0.2
18:0	11.2±0.5	12.2±0.6	8.2±0.3	7.6±0.3	8.8±1.5	7.3±0.4
18:1	34.5±1.0	28.0±1.6	33.9±1.0	37.1±1.5	38.3±0.1	38.3±1.7
18:2	12.1±1.2	11.2±0.2	18.4±0.1	18.3±0.5	20.3±0.5	18.8±1.4
18:3	0.9±0.07	0.77±0.006	1.24±0.08	1.07±0.09	1.00±0.08	1.02±0.1
20:4	4.3±0.7	7.7±1.2	3.6±0.2	2.2±0.3	1.5±0.2	1.7±0.3
22:6	8.9±0.6	9.1±1.9	4.8±0.6	2.6±0.3	0.6±0.2	1.0±0.3

Table 3.16 : Fatty acid composition (% w/w of total fatty acid; means ± S.E.M. of 4 embryos) of whole embryo phospholipid from experiment 1.

	Day 6	11	13	15	18	22
16:0	32.7±1.4	30.5±0.3	26.9±0.5	27.0±0.5	27.1±1.5	29.2±0.4
18:0	14.5±0.3	16.0±0.6	16.7±0.4	18.1±0.3	21.6±0.5	17.9±0.6
18:1	19.6±0.5	18.8±0.3	18.0±0.4	17.6±0.3	17.4±0.4	14.7±1.5
18:2	3.4±0.2	4.7±0.2	6.8±0.2	9.4±0.09	11.8±0.6	9.8±0.9
18:3	0.35±0.01	0.20±0.07	0.32±0.02	0.34±0.04	0.36±0.04	0.20±0.07
20:4	12.9±0.3	16.4±0.8	17.3±0.4	15.3±0.2	14.2±1.1	16.1±0.7
22:6	13.3±0.2	10.0±1.0	9.8±0.3	9.5±0.2	7.2±0.5	9.0±0.4

Table 3.17 : Fatty acid composition (% w/w of total fatty acid; means ± S.E.M. of 4 embryos) of whole embryo chick cholesteryl ester from experiment 1.

	Day 6	11	13	15	18	22
16:0	17.9±1.8	17.8±2.4	11.3±1.5	8.5±0.7	7.0±0.7	9.5±1.1
18:0	12.3±0.3	7.7±0.6	5.5±0.3	4.2±0.2	5.2±0.8	5.7±0.5
18:1	35.3±0.9	41.3±4.7	49.3±1.6	59.2±2.6	64.7±1.2	58.6±1.4
18:2	14.7±0.6	16.9±1.0	21.4±0.7	21.2±0.6	15.1±1.1	17.1±0.8
18:3	0.2±0.1	0.30±0.07	0.57±0.06	0.50±0.09	0.47±0.09	0.70±0.01
20:4	9.6±0.6	9.1±0.4	6.6±1.2	5.2±0.3	4.2±0.2	5.2±1.1
22:6	14.5±0.7	6.5±1.8	3.2±0.4	1.2±0.3	1.0±0.4	1.8±0.1

Table 3.18: Fatty acid composition (% w/w of total fatty acid; means ± S.E.M. of 4 embryos) of whole embryo triacylglycerol from experiment 2.

	Day 12	13	14	15	18	19	22
16:0	27.0 ± 1.1	29.6 ± 0.6	28.1 ± 0.6	28.8 ± 0.8	29.8 ± 0.6	29.6 ± 1.2	29.5 ± 0.5
18:0	9.2 ± 0.4	8.3 ± 0.2	3.2 ± 0.1	7.6 ± 0.3	7.3 ± 0.3	7.0 ± 0.2	7.9 ± 0.1
18:1	31.7 ± 1.2	33.8 ± 0.8	35.0 ± 1.2	36.9 ± 0.8	39.8 ± 0.8	39.4 ± 1.5	41.4 ± 0.8
18:2	11.0 ± 0.7	11.9 ± 0.8	13.6 ± 0.7	13.0 ± 0.5	13.5 ± 0.3	13.8 ± 1.0	14.3 ± 0.2
18:3	0.79±0.08	0.71±0.02	0.8 ± 0.02	0.82±0.05	0.92±0.03	0.8 ± 0.09	0.81±0.05
20:4	3.0 ± 0.4	2.5 ± 0.1	2.1 ± 0.2	1.9 ± 0.2	1.10±0.08	1.0 ± 0.1	0.91±0.05
22:6	10.0 ± 1.5	8.5 ± 0.4	8.2 ± 0.4	6.4 ±0.5	2.7 ± 0.7	2.4 ± 0.2	1.1 ± 0.1

Table 3.19 : Fatty acid composition (% w/w of total fatty acid; means ± S.E.M. of 4 embryos) of whole embryo phospholipid from experiment 2.

	Day 12	13	14	15	18	19	22
16:0	29.6 ± 0.1	29.7 ± 0.4	29.3 ± 0.3	32.8 ± 1.4	31.3 ± 0.6	31.3 ± 0.7	29.1 ± 1.5
18:0	15.4 ± 0.2	15.8 ± 0.2	16.1 ± 0.3	18.0 ± 1.3	17.0 ± 1.1	16.7 ± 0.2	21.2 ± 0.9
18:1	18.9 ± 0.2	18.8 ± 0.4	19.2 ± 0.6	18.5 ± 0.4	19.0 ± 0.9	18.4 ± 0.6	19.4 ± 0.4
18:2	5.2 ± 0.3	5.9 ± 0.3	7.4 ± 0.2	7.5 ± 0.7	9.0 ± 1.0	10.7 ± 0.8	10.0 ± 0.2
18:3	0.25±0.01	0.26±0.01	0.33±0.08	0.30±0.03	0.27±0.02	0.19±0.02	0.28±0.02
20:4	12.5± 0.3	12.1± 0.2	11.8± 0.5	9.5 ± 0.9	9.9 ± 0.2	10.2± 0.3	9.1 ± 0.5
22:6	13.0 ± 0.3	12.6 ± 0.2	11.6 ± 0.3	11.6 ± 0.2	10.0 ± 0.4	9.4 ± 0.04	8.6 ± 0.2

Table 3.20 : Fatty acid composition (% w/w of total fatty acid; means ± S.E.M. of 4 embryos) of whole embryo cholesteryl ester from experiment 2.

	Day 12	13	14	15	18	19	22
16:0	17.0 ± 1.0	14.7 ± 0.6	13.7 ± 1.3	12.6 ± 0.7	10.2 ± 0.5	8.8 ± 0.6	5.7 ± 0.3
18:0	6.2 ± 1.1	6.4 ± 0.5	4.9 ± 0.2	6.2 ± 0.2	6.6 ± 0.8	5.5 ± 0.3	4.6 ± 0.2
18:1	40.7 ± 0.3	43.0 ± 0.6	52.0 ± 1.8	56.5 ± 0.9	60.5 ± 1.9	62.6 ± 2.4	69.2 ± 2.3
18:2	16.6 ± 0.8	16.6 ± 0.7	19.1 ± 1.2	16.0 ± 0.7	13.6 ± 0.6	13.7 ± 1.1	11.9 ± 0.2
18:3	0.45±0.07	0.48±0.02	0.50±0.06	0.6±0.07	0.51±0.05	0.46±0.06	0.38±0.02
20:4	7.8 ± 1.1	5.4 ± 0.5	3.6 ± 0.2	3.2 ± 0.4	1.9 ± 0.2	2.17±0.05	2.03 ± 0.2
22:6	8.2 ± 0.6	9.7 ± 1.7	6.0 ± 0.3	2.6 ± 0.6	4.2 ± 1.0	2.1 ± 0.4	2.5 ± 0.8

Figure 3.7: Accumulation of fatty acids in the lipid of the whole embryo. The changes in the levels of oleic acid (○), AA (●) and DHA (□) are shown during incubation of the "small" (a) and "normal" (b) eggs. Values are expressed as percentage of the amounts in the initial yolks prior to incubation and are the means of 4 replicates. Comparison between oleic and DHA values : $^{a}P<0.001$, $^{b}P<0.002$, $^{c}P<0.02$.

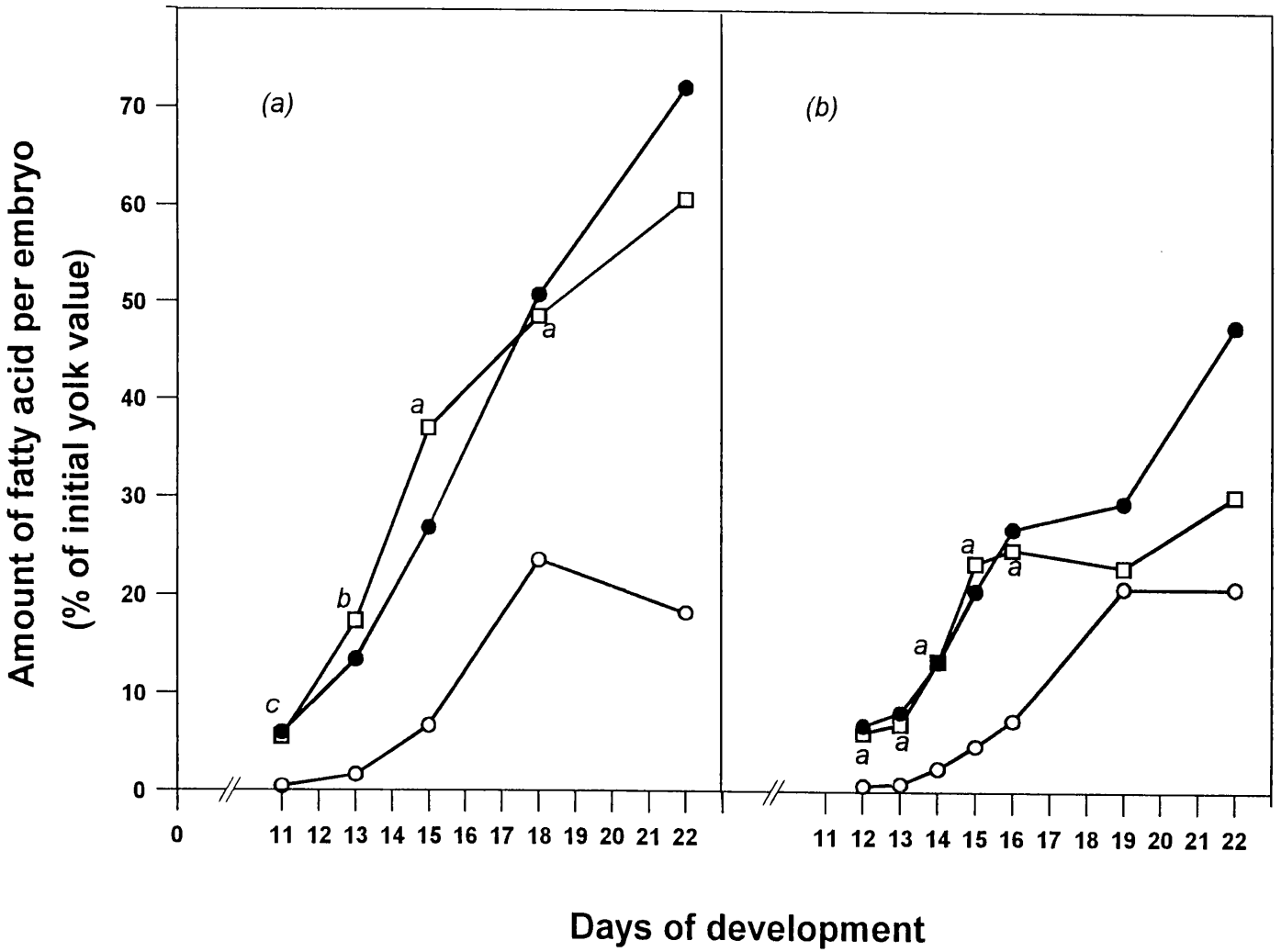

Amount of fatty acid per embryo
(% of initial yolk value)
(a)
(b)
70
60
50
40
30
20
10
0
0
11 12 13 14 15 16 17 18 19 20 21 22
11 12 13 14 15 16 17 18 19 20 21 22
Days of development

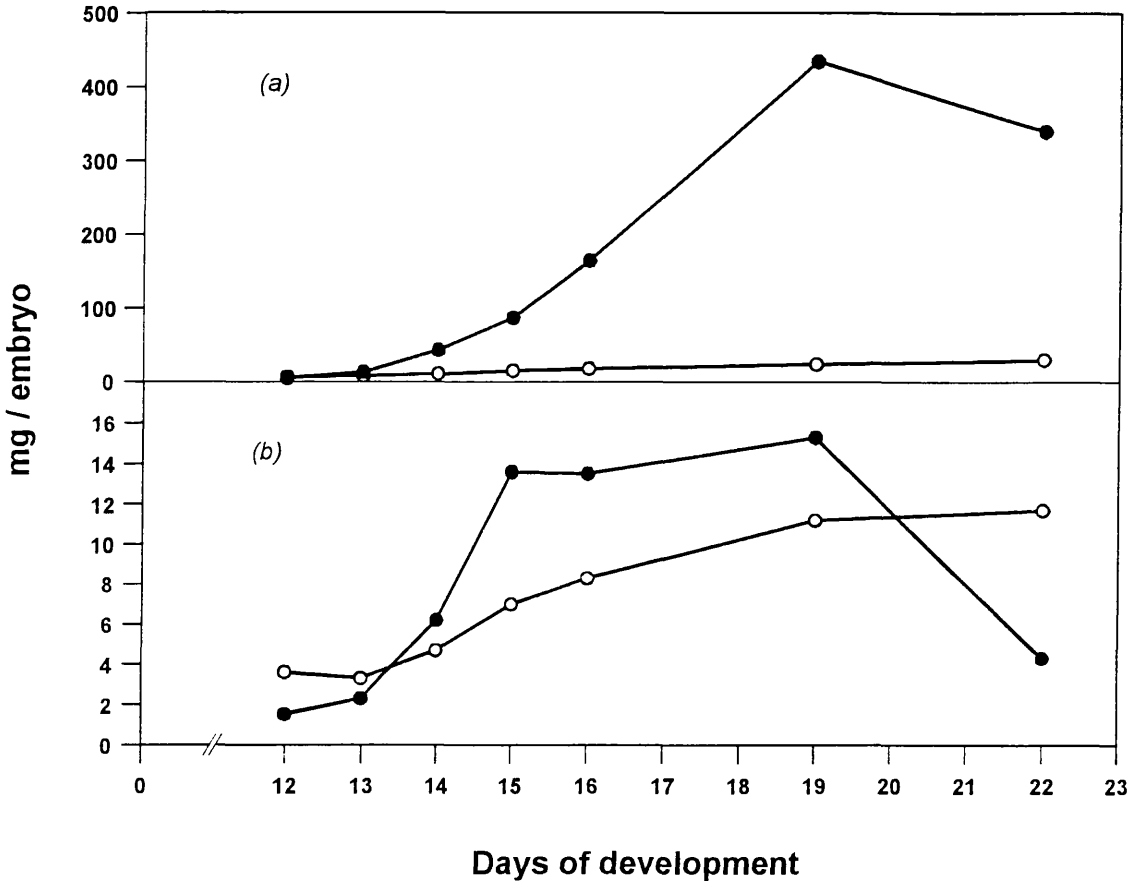
Table 3.21 : Recovery of yolk lipids of the newly hatched chick. The amount of each fatty acid present in total lipid per day-old chick is expressed as a percentage of the amount transferred from the initial yolk between the first day of embryogenesis and the first day post-hatching. The values used to calculate the recoveries were the means of 4 initial yolks and 4 day-old chicks, and the S.E.M. of the replicates were in all cases less than 12% of the means.

	Amount in lipids of day-old chick as % of that transferred from yolk	
	Experiment 1	Experiment 2
16:0	20.5	25.0
18:0	26.5	26.2
18:1	19.0	22.4
18:2	25.3	26.2
18:3	17.2	21.6
20:4	74.6	50.0
22:6	60.6	30.8

Table 3.22: Recoveries of triacylglycerol and phospholipid in the newly hatched chick. The amounts of total lipid, TG and PL per day old chick are expressed as percentages of the amounts of the respective lipid classes transferred from the initial yolk between the first day of embryogenesis and the first day post hatching. The values used to calculate the recoveries were the means of 4 initial yolks and 4 day-old chicks, and the standard error were in all cases less than 12% of the means. The initial yolk levels of triacylglycerol were 4004 ± 70 and 2622 ± 70 mg/yolk for "normal" (experiment 2) and "small" (experiment 1) eggs respectively. The respective initial phospholipid levels were 1166 ± 54 and 836 ± 32 mg/yolk.

	Amount in lipids of day-old chick as % of that transferred from yolk	
	Experiment 1	Experiment 2
Total lipids	30.0	30.4
Triacylglycerol	14.6	22.6
Phospholipid	25.8	18.5

Figure 3.8: Levels of 18:1 and DHA in TG and PL of the embryo. The amount (mg/embryo) of 18:1 (a) and DHA (b) incorporated into TG (●) and PL (○) of the whole embryo are shown during incubation of the "normal" eggs. Values are the means of measurements on 4 embryos at each time-point and S.E.M.'s were in all cases less than 10 % of the means.


500
400
300
200
100
0
(a)
16
14
12
10
8
6
4
2
0
(b)
0
12
13
14
15
16
17
18
19
20
21
22
23
mg / embryo
Days of development

3.1.4.Plasma lipid composition during development

Plasma samples were collected during experiment 2, and also during an additional experiment (experiment 3). The eggs used for experiment 3 were obtained from a 40 week old laying flock and initially weighed 64.3 ±1.1 (n=12) g and contained 5.94 ± 0.09 (n=10) g of total lipid per yolk.

The concentration of total lipids in the embryonic circulation (Experiment 3) increased from day 12 to day 18 of incubation and then decreased prior to and after hatching (Table 3.23). The main plasma lipid class from day 12 to day 14 was triacylglycerol, and this class thereafter decreased continuously, reaching very low levels just after hatching. From day 14 onwards, cholesteryl ester was the most abundant class in the circulation of the embryonic and neonatal chick (Tables 3.24-3.25) reflecting a possible accumulation in the circulation of remnant lipoproteins and/or transfer of lipoprotein surface components to high density lipoprotein (Figure 1.5 in *1.3.4*).

High proportionate levels of DHA were present in plasma TG, especially between days 12 to 18. The level of DHA in plasma TG was maximal (up to 13.5 % w/w of fatty acids) at around days 12 to 14, decreasing to lower levels around the hatching period (Tables 3.26 and 3.30). In contrast, much lower levels of DHA were present in plasma PL (Tables 3.27 and 3.31). Thus it appears that, following lipid uptake into the yolk sac membrane, DHA is transferred from yolk PL and incorporated into TG for secretion as plasma lipoprotein.

Although significant levels of AA were present in plasma TG, much higher levels of the fatty acid (up to nearly 20 % w/w of fatty acids) were found in the PL fraction (Tables 3.26, 3.27, 3.30 and 3.31). It therefore appears that, in the yolk sac membrane, DHA and AA are differentially distributed between the lipid classes into TG and PL respectively, prior to lipoprotein assembly. Although some DHA was present in plasma CE, the level was never higher than about 4% (w/w). Although FFA was only a minor component of plasma lipid (Tables 3.24 and 3.25), very high proportions of DHA (up to 17% w/w) were in this fraction (Tables 3.29 and 3.33).

In conclusion, the plasma lipids exhibited a number of specific compositional features, particularly in relation to DHA distribution, and presumably reflecting the characteristics of a highly specialised mechanism for the delivery of long chain polyunsaturates to particular tissues.

Table 3.23: Concentration of total lipid in plasma of the embryo or chick (experiment 3). Values are the means ± S.E.M. of 4 plasma samples at each stage. Days 22 and 25 are equivalent to 1 and 4 days after hatching, respectively.

Day	Lipid concentration (mg/ml)
12	2.2 ± 0.1
13	3.4 ± 0.1
14	4.6 ± 0.1
15	6.4 ± 0.2
18	19.5 ± 1.7
19	12.7 ± 0.4
22	9.5 ± 0.2
25	5.0 ± 0.1

Table 3.24: Plasma lipid composition (lipid class as % w/w of total lipid) during development (experiment 2). Values are the means ± S.E.M. of 4 plasma samples at each stage.

% total lipid	Day 12	13	14	15	16	19	22
CE	20 ± 0.2	22.4 ± 0.1	29.8 ± 0.2	32.8 ± 0.3	39.4 ± 0.5	42.4 ± 1.5	45.4 ± 1.1
TG	40.2 ± 1.1	35.8 ± 0.6	30.2 ± 0.6	26.7 ± 0.6	23.5 ± 0.7	18.0 ± 0.8	8.2 ± 0.9
FFA	1.4 ± 0.1	2.1 ± 0.5	1.4 ± 0.1	1.5 ± 0.1	1.5 ± 0.3	3.6 ± 0.6	2.0 ± 0.3
CHOL	10.3 ± 0.6	12.2 ± 0.3	9.2 ± 0.3	10.3 ± 0.2	9.2 ± 0.8	6.1 ± 0.9	8.2 ± 0.9
PL	28.4 ± 0.6	27.5 ± 0.5	29.4 ± 0.4	28.5 ± 0.8	26.5 ± 0.8	29.8 ± 1.6	36.2 ± 2.8

Table 3.25: Plasma lipid composition (lipid class as % w/w of total lipid) during development (experiment 3). Details as for Table 3.23.

% total lipid	Day 12	13	14	15	18	19	22	25
CE	18.7 ± 1.0	21.0 ± 0.4	24.5 ± 0.3	33.2 ± 0.5	36.5 ± 1.4	41.4 ± 0.6	45.5 ± 1.4	38.9 ± 0.6
TG	42.8 ± 0.5	38.7 ± 0.5	33.8 ± 0.9	26.8 ± 0.4	31.4 ± 2.1	17.4 ± 0.4	2.3 ± 0.8	9.1 ± 1.2
FFA	2.3 ± 0.3	1.6 ± 0.1	1.8 ± 0.2	2.4 ± 0.4	2.7 ± 1.0	2.3 ± 0.6	5.3 ± 1.9	6.9 ± 1.6
CHOL	12.5 ± 1.1	12.7 ± 0.2	12.2 ± 1.5	10.9 ± 0.4	6.3 ± 1.1	11.3 ± 0.6	13.4 ± 0.5	11.2 ± 0.3
PL	25.6 ± 1.1	27.5 ± 0.6	27.8 ± 1.7	26.7 ± 0.5	23.2 ± 1.1	27.7 ± 0.7	33.6 ± 0.6	34.0 ± 0.5

Table 3.26: Fatty acid composition (% w/w of total fatty acid) of plasma triacylglycerol during development (experiment 2). Values are means ± S.E.M., n=4.

Fatty acids (%)	Day 12	13	14	15	16	19	22
16:0	26.7 ± 0.4	25.8 ± 0.3	25.4 ± 0.4	25.9 ± 0.9	27.5 ± 0.	24.7 ± 1.2	21. ± 0.7
16:1	2.6 ± 0.04	ND	ND	2.2 ± 0.8	3.2 ± 0.06	3.5 ± 0.4	ND
18:0	8.0 ± 0.1	7.5 ± 0.1	7.3 ± 0.04	7.7 ± 0.1	6.7 ± 0.2	7.0 ± 0.2	10.8 ± 0.3
18:1	32.3 ± 0.4	34.9 ± 0.6	36.3 ± 0.7	38.3 ± 1.0	37.5 ± 0.8	44.0 ± 0.7	43.7 ± 1.0
18:2	13.3 ± 0.2	13.9 ± 0.2	15.5 ± 0.3	15.8 ± 0.3	16.6 ± 0.1	16.6 ± 0.7	15.1 ± 0.1
18:3	0.7 ± 0.05	0.8 ± 0.02	0.9 ± 0.02	0.9 ± 0.07	0.9 ± 0.03	0.8 ± 0.2	0.5 ± 0.1
20:4	3.7 ± 0.06	4.0 ± 0.2	2.9 ± 0.1	2.6 ± 0.1	2.0 ± 0.05	0.7 ± 0.2	2.8 ± 0.1
20:5	0.7 ± 0.02	0.6 ± 0.04	0.4 ± 0.01	0.3 ± 0.02	0.3 ± 0.07	ND	ND
22:5	0.5 ± 0.02	0.5 ± 0.03	0.7 ± 0.03	0.5 ± 0.05	0.3 ± 0.05	0.2 ± 0.02	0.8 ± 0.05
22:6	11.8 ± 0.1	11.1 ± 0.5	9.2 ± 0.4	6.3 ± 1.0	5.7 ± 0.3	1.6 ± 0.4	4.8 ± 1.0

Table 3.27: Fatty acid composition (% w/w of total fatty acids) of plasma phospholipid during development (experiment 2). Values are means ± S.E.M., n=4.

Fatty acids (%)	Day 12	13	14	15	16	19	22
16:0	31.6 ± 0.1	30.9 ± 0.8	30.6 ± 0.5	31.9 ± 0.9	31.7 ± 0.4	25.2 ± 1.2	23.0 ± 0.7
16:1	ND	ND	ND	ND	ND	ND	ND
18:0	15.8 ± 0.2	16.0 ± 0.3	15.8 ± 0.3	15.8 ± 0.2	14.6 ± 0.3	17.1 ± 0.3	19.2 ± 0.5
18:1	12.7 ± 0.1	12.6 ± 1.0	13.0 ± 0.1	14.4 ± 0.2	13.8 ± 0.4	16.6 ± 0.3	16.2 ± 0.2
18:2	16.6 ± 0.1	19.5 ± 0.7	25.1 ± 0.5	26.0 ± 0.5	28.2 ± 1.2	24.4 ± 0.1	21.8 ± 0.1
18:3	ND	ND	ND	ND	ND	ND	0.2 ± 0.02
20:4	19.5 ± 0.1	18.4 ± 0.9	13.0 ± 0.2	9.9 ± 0.3	9.9 ± 0.5	13.5 ± 0.4	15.2 ± 0.5
20.5	0.3 ± 0.03	0.3 ± 0.04	ND	0.2 ± 0.02	ND	0.2 ± 0.02	0.4 ± 0.03
22:5	0.2 ± 0.05	0.07 ± 0.01	ND	ND	ND	0.2 ± 0.02	0.3 ± 0.02
22:6	2.4 ± 0.1	2.2 ± 0.1	1.7 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	2.3 ± 0.07	3.3 ± 0.5

Table 3.28: Fatty acid composition (% w/w of total fatty acids) of plasma cholesteryl ester during development (experiment 2). Values are means ± S.E.M., n=4.

Fatty acids (%)	Day 12	13	14	15	16	19	22
16:0	15.6 ± 0.1	15.1 ± 0.2	9.5 ± 0.1	7.7 ± 0.1	6.8 ± 0.1	7.7 ± 0.3	8.8 ± 0.2
[illegible]:1	0.7 ± 0.2	0.8 ± 0.05	1.0 ± 0.1	0.8 ± 0.1	1.0 ± 0.06	0.8 ± 0.2	0.7 ± 0.1
18:0	3.5 ± 0.1	3.1 ± 0.06	3.2 ± 0.02	3.2 ± 0.04	3.1 ± 0.04	4.0 ± 0.4	3.4 ± 0.05
18:1	42.6 ± 0.3	43.1 ± 0.2	52.4 ± 0.2	58.1 ± 0.3	60.2 ± 0.06	50.9 ± 0.8	46.5 ± 0.1
18:2	27.7 ± 0.1	29.1 ± 0.1	28.2 ± 0.1	26.2 ± 0.04	24.2 ± 0.1	28.2 ± 0.4	32.1 ± 0.2
18:3	0.6 ± 0.01	0.6 ± 0.02	0.9 ± 0.01	0.9 ± 0.1	1.0 ± 0.01	0.9 ± 0.05	0.9 ± 0.01
20:4	4.8 ± 0.05	4.8 ± 0.4	2.5 ± 0.01	1.9 ± 0.1	1.7 ± 0.05	3.5 ± 0.1	4.2 ± 0.1
20:5	0.9 ± 0.02	0.7 ± 0.06	0.6 ± 0.02	0.5 ± 0.01	0.6 ± 0.05	0.7 ± 0.2	1.1 ± 0.03
22:6	3.5 ± 0.1	2.5 ± 0.2	1.6 ± 0.01	1.0 ± 0.06	1.3 ± 0.08	2.3 ± 0.2	1.9 ± 0.05

Table 3.29: Fatty acid composition (% w/w of total fatty acids) of plasma free fatty acids during development (experiment 2). Values are means ± S.E.M., n=4.

Fatty acids (%)	Day 12	13	14	15	16	19	22
16:0	24.2 ± 0.9	31.2 ± 1.0	24.4 ± 0.7	24.0 ± 0.8	25.8 ± 2.9	24.7 ± 3.0	22.8 ± 0.7
16:1	1.8 ± 0.1	ND	1.3 ± 0.4	ND	ND	ND	ND
18:0	18.2 ± 0.5	17.8 ± 1.6	16.3 ± 0.5	16.0 ± 0.8	19.2 ± 0.4	27.6 ± 1.0	21.7 ± 0.9
18:1	21.7 ± 0.6	28.4 ± 0.6	27.8 ± 0.8	30.5 ± 0.8	27.3 ± 0.5	34.2 ± 1.8	34.4 ± 1.2
18:2	8.3 ± 0.02	9.2 ± 0.5	11.6 ± 0.2	11.5 ± 0.7	11.6 ± 0.3	8.0 ± 0.4	11.6 ± 0.5
18:3	0.4 ± 0.02	0.7 ± 0.01	0.8 ± 0.06	ND	ND	ND	1.0 ± 0.04
20:3	ND	1.1 ± 0.2	0.6 ± 0.1	ND	ND	2.6 ± 0.8	ND
20:4	5.1 ± 0.2	3.8 ± 0.2	4.3 ± 0.4	4.6 ± 0.2	3.6 ± 0.6	2.8 ± 0.5	2.9 ± 0.3
20:5	0.7 ± 0.3	ND	0.8 ± 0.1	ND	ND	ND	ND
22:6	14.0 ± 0.8	5.8 ± 2.0	10.2 ± 1.0	9.4 ± 0.4	9.6 ± 0.4	ND	4.2 ± 0.3

Table 3.30: Fatty acid composition (% w/w of total fatty acids) of plasma triacylglycerol during development (experiment 3). Values are means ± S.E.M., n=4.

Fatty acids (%)	Day 12	13	14	15	18	19	22	25
16:0	25.6 ± 0.6	26.2 ± 0.3	25.7 ± 0.2	25.4 ± 0.3	27.8 ± 0.8	29.1 ± 0.2	29.5 ± 1.6	25.5 ± 0.5
16:1	2.2 ± 0.02	2.2 ± 0.02	2.5 ± 0.06	2.4 ± 0.07	2.4 ± 0.2	2.8 ± 0.1	ND	4.8 ± 0.2
18:0	8.0 ± 0.3	8.2 ± 0.01	8.1 ± 0.06	8.2 ± 0.1	7.9 ± 0.1	7.7 ± 0.1	11.2 ± 1.6	6.5 ± 0.2
18:1	34.4 ± 0.8	34.4 ± 0.3	36.4 ± 0.1	39.1 ± 0.3	40.7 ± 0.7	42.0 ± 0.2	39.8 ± 0.3	39.1 ± 0.5
18:2	10.9 ± 0.6	10.2 ± 0.1	10.6 ± 0.4	10.6 ± 0.1	11.3 ± 0.2	11.7 ± 0.2	11.1 ± 0.7	14.9 ± 0.7
18:3	0.5 ± 0.01	0.4 ± 0.07	0.7 ± 0.01	0.6 ± 0.01	0.7 ± 0.02	0.7 ± 0.02	ND	2.3 ± 0.1
20:4	3.5 ± 0.5	3.4 ± 0.1	3.0 ± 0.03	2.6 ± 0.07	1.6 ± 0.1	1.1 ± 0.05	1.6 ± 0.3	0.7 ± 0.1
22:6	11.5 ± 0.9	13.5 ± 0.3	10.6 ± 0.5	10 1 ± 0.2	6.4 ± 0.3	3.5 ± 0.1	1.4 ± 0.2	2.0 ± 0.1

Table 3.31: Fatty acid composition (% w/w of total fatty acids) of plasma phospholipid during development (experiment 3). Values are means ± S.E.M., n=4.

Fatty acids (%)	Day 12	13	14	15	18	19	22	25
16:0	32.7 ± 0.3	32.6 ± 0.1	24.1 ± 0.2	31.4 ± 0.7	27.8 ± 1.3	27.7 ± 0.2	23.9 ± 0.4	24.9 ± 0.1
16:1	0.2 ± 0.01	0.6 ± 0.02	1.3 ± 0.1	0.4 ± 0.3	0.5 ± 0.1	0.7 ± 0.02	0.8 ± 0.04	1.9 ± 0.1
18:0	15.8 ± 0.3	15.9 ± 0.05	9.9 ± 0.2	15.7 ± 0.3	17.0 ± 0.5	16.8 ± 0.1	21.2 ± 0.1	19.9 ± 0.2
18:1	15.3 ± 0.3	14.1 ± 0.03	32.4 ± 0.3	16.5 ± 0.2	17.5 ± 0.2	15.2 ± 0.2	14.2 ± 0.02	17.2 ±0.06
18:2	13.9 ± 0.1	17.4 ± 0.1	16.1 ± 0.2	22.4 ± 0.1	24.2 ± 0.5	22.9 ± 0.4	18.3 ± 0.06	19.7 ±0.04
18:3	ND	0.1 ± 0.01	0.5 ± 0.01	0.1 ± 0.01	ND	0.2 ± 0.02	0.3 ± 0.04	0.6 ± 0.01
20:4	17.8 ± 0.1	15.4 ± 0.1	7.1 ± 0.2	10.7 ± 0.3	10.9 ± 1.1	12.9 ± 0.7	14.4 ± 0.4	5.0 ± 0.03
22:6	3.1 ± 0.1	2.3 ± 0.03	7.0 ± 0.3	1.8 ± 0.1	1.3 ± 0.2	2.3 ± 0.1	5.1 ± 0.1	5.1 ± 0.07

Table 3.32: Fatty acid composition (% w/w of total fatty acids) of plasma cholesteryl ester during development (experiment 3). Values are means ± S.E.M., n=4.

Fatty acids (%)	Day 12	13	14	15	18	19	22	25
16:0	17.6 ± 0.1	14.2 ± 0.2	11.8 ± 0.1	9.2 ± 0.4	7.6 ± 0.3	8.1 ± 0.05	10.2 ± 0.1	13.0 ± 0.2
16:1	0.9 ± 0.07	1.2 ± 0.1	1.3 ± 0.01	1.3 ± 0.06	1.3 ± 0.07	1.5 ± 0.01	1.5 ± 0.04	4.0 ± 0.1
18:0	3.1 ± 0.1	3.0 ± 0.1	3.0 ± 0.06	3.3 ± 0.1	4.1 ± 0.3	3.4 ± 0.02	2.9 ± 0.05	2.5 ± 0.3
18:1	44.5 ± 0.4	48.3 ± 0.6	53.6 ± 0.2	59.6 ± 0.4	65.4 ± 0.1	56.7 ± 0.2	43.1 ± 0.1	35.4 ± 0.6
18:2	22.0 ± 0.2	23.9 ± 0.2	23.4 ± 0.06	21.0 ± 0.3	16.8 ± 0.4	22.4 ± 0.05	31.9 ± 0.06	34.3 ± 0.6
18:3	0.3 ± 0.02	0.4 ± 0.01	0.5 ± 0.01	0.5 ± 0.01	0.6 ± 0.01	0.7 ± 0.01	0.7 ± 0.01	2.1 ± 0.3
20:4	4.7 ± 0.2	4.2 ± 0.3	2.8 ± 0.1	2.1 ± 0.03	1.7 ± 0.02	3.1 ± 0.1	4.0 ± 0.04	1.6 ± 0.1
20:5	1.1 ± 0.02	1.0 ± 0.1	0.9 ± 0.03	0.7 ± 0.02	0.8 ± 0.05	1.3 ± 0.03	2.1 ± 0.05	2.2 ± 0.1
22:6	4.0 ± 0.6	2.6 ± 0.4	2.5 ± 0.04	2.3 ± 0.08	1.7 ± 0.07	2.6 ± 0.04	3.1 ± 0.07	2.5 ± 0.2

Table 3.33: Fatty acid composition (% w/w of total fatty acids) of plasma free fatty acids during development (experiment 3). Values are means ± S.E.M., n=4.

Fatty acids (%)	Day 12	13	14	15	18	19	22	25
16:0	30.7 ± 1.1	26.3 ± 0.8	27.5 ± 1.0	22.1 ± 1.3	31.3 ± 4.5	32.4 ± 1.4	24.6 ± 1.8	24.0 ± 0.4
16:1	ND	ND	ND	1.1 ± 0.2	ND	ND	1.9 ± 0.2	3.3 ± 0.6
18:0	16.4 ± 1.1	15.6 ± 0.7	20.1 ± 0.5	18.5 ± 1.8	29.4 ± 2.3	24.8 ± 1.9	14.0 ± 1.0	9.5 ± 1.4
18:1	23.4 ± 1.7	24.2 ± 1.1	28.7 ± 0.7	27.7 ± 1.6	26.3 ± 1.5	26.9 ± 1.7	38.4 ± 0.5	32.4 ± 1.0
18:2	4.7 ± 0.1	6.3 ± 0.1	6.9 ± 0.2	7.1 ± 0.6	5.6 ± 0.3	8.8 ± 1.4	11.1 ± 0.5	18.5 ± 0.5
18:3	ND	0.4 ± 0.04	ND	ND	ND	ND	0.9 ± 0.08	3.3 ± 0.2
20:3	5.4 ± 0.8	3.1 ± 0.1	3.1 ± 0.2	2.5 ± 0.5	7.7 ± 2.1	4.0 ± 0.3	0.7 ± 0.1	ND
20:4	3.0 ± 0.5	4.8 ± 0.5	3.0 ± 0.1	4.0 ± 0.6	ND	ND	1.7 ± 0.4	0.9 ± 0.07
20:5	ND	1.1 ± 0.2	ND	ND	ND	ND	ND	2.0 ± 0.2
22:6	12.9 ± 0.2	17.1 ± 1.1	10.1 ± 0.6	15.7 ± 1.7	ND	ND	5.2 ± 1.1	4.2 ± 0.6

3.1.5. Fatty acid composition of adipose tissue TG during development

The development of adipose tissue in the chick embryo was studied in 2 experiments; experiment 2, as described previously (section *3.1.1*) using "normal" size eggs from 33 week old laying birds, and an additional experiment using "small" eggs from a 24 week old laying flock. Compositional parameters for these "small" eggs are given in Table 3.34. The same parameters for the "normal" size eggs have been previously described (Table 3.1) but are included in Table 3.34 to facilitate comparison. The data in Table 3.34 indicate that the amount of total lipid available to the embryo was approximately 1.7-fold greater in the "normal" size compared to the "small" eggs.

Figure 3.9 indicates that the embryo fresh weights were greater during development in the "normal" size eggs at all stages of development. Prior to hatching, at day 19, there was a 1.3-fold difference in embryo weight between the 2 experiments.

The development of adipose tissue in the embryo is illustrated in Figure 3.10. Adipose tissue was undetectable to the unaided eye prior to day 12. After this time, the amount of adipose TG increased rapidly, reaching a maximal level just prior to hatching at day 19. Over the hatching period, between days 19 and 22, there was no further growth of the tissue and in fact a significant decrease in the adipose TG content was observed in both experiments. The amount of TG deposited in the adipose tissue was proportional to the initial yolk lipid content (Table 3.34 and Figure 3.10); thus at day 19 the amount of adipose TG was 1.5-fold greater in the "normal" compared to the "small" egg experiment. The results in Table 3.35 indicate that the reduced amount of adipose tissue in the "small" egg experiment was not simply due to the reduction in embryo size, since the adipose TG content as a percentage of whole embryo weight was also generally lower. Thus the initial egg size and lipid content affect the subsequent weight of the embryo plus a further specific effect on the amount of adipose tissue. The amount of lipid transferred from the yolk on the examined days of incubation ("normal" size egg experiment) are compared to the amount of TG deposited in the adipose tissue. The results in Figure 3.11 indicate that, during embryogenesis, the accumulation of TG in adipose tissue is closely related to the transfer of lipid from the yolk. However, during the peri-hatching period (day 19 of embryo development to 1 day after hatching), this relationship breaks down as accumulation of adipose TG ceases (in fact there is some loss of lipid from the tissue over this period i.e. mobilisation) in spite of the continued transfer of lipid from the retracted yolk.

The fatty acid compositions (%, w/w) of adipose TG are given in Tables 3.36 and 3.37. High proportions (up to 7.2 %) of DHA were present in the TG at days 12 and 14 decreasing to lower levels by hatching. Significant levels of 20:4 (up to 4%) were also present at days 12 and 14, again decreasing at the later stages. The major fatty acid in adipose TG was 18:1 at all stages. The absolute amounts of 18:1, in terms

of mg of the fatty acid in TG per adipose depot, are shown in Figure 3.12. The amount of 18:1 increased continuously to reach a maximum value just prior to hatching and the levels were dependent on the initial yolk lipid content. Between days 19 and 22, the amount of 18:1 remained the same ("normal" size eggs) or decreased ("small" eggs). Thus the pattern of change in the amount of adipose TG was closely reflected in the amounts of constituent 18:1. Furthermore, the changes in the absolute amounts (mg in TG/depot) of 16:0, 18:0, 18:2, 18:3 and 20:4 (data not shown) followed a pattern which was essentially identical to that shown for 18:1 in Figure 3.12.

In marked contrast, the changes in the absolute amount (mg in TG/depot) of DHA in adipose TG followed a different pattern from the other fatty acids (Figure 3.13). Firstly, the amount of DHA did not increase continuously up to hatching but instead reached a plateau level by day16. Secondly, there was a dramatic decrease in the DHA content of the tissue over the hatching period, suggesting a preferential mobilisation of this fatty acid.

Thus whereas the amounts of oleic acid mobilised perinatally from the tissue in the 2 experiments amounted to 1 and 20% of the day 19 values (Figure 3.12), the respective mobilisations of DHA over this period amounted to 70 and 85% of the pre-hatch adipose DHA stores (Figure 3.13).

In an additional experiment (experiment 3 as described in section *3.1.4.*), measurements on adipose tissue composition were performed on birds which were maintained to day 4 after hatching with free access to food. The percentage fatty acid compositions are shown in Table 3.38. The TG contained a high proportion of DHA at day 15, and this value decreased dramatically at the later developmental stages. The absolute amounts (mg in TG per depot) of the individual fatty acids are shown in Figures 3.14 a and b. The amounts of 16:0, 18:0, 18:1, 18:2 and 20:4 increased continuously until hatching, with little further change during the first 4 post-hatch days. However, in agreement with the previous study (Figure 3.13), the amount of DHA decreased over the hatching period, with a further decrease by 4 days post hatch (Figure 3.14 b). In this experiment, the amount of DHA in adipose TG was considerably higher than in the previous experiments (Figure 3.13 and 3.14 b) reflecting the differences in the initial yolk DHA contents (Tables 3.1 and 3.34). Also, over the hatching period (day 18 to 22 in experiment 3), the proportion of adipose DHA mobilised was less than in the previous studies.

However, the absolute amounts of DHA released from the tissue were very similar (approximately 4 mg) in the 3 experiments.

In conclusion, the results in this section suggest that DHA is preferentially mobilised from adipose TG during the peri-hatching and neonatal periods.

Table 3.34: Characteristics of eggs prior to incubation for the adipose tissue study. Results are the means ± S.E.M. of measurements on 4 eggs with the exception of egg weights where n=10. For convenience, the eggs from the 33 and 24 week old flocks are colloquially referred to in the text as "normal" size and "small" respectively. Significant differences for values on the same line (between experiments) are illustrated with superscript character $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$, $^{n}P>0.05$ not significant.

	Age of parental flock (weeks)	
	24	33
Egg weight (g)	48.4 ± 0.7	62.2 ± 0.7 ^{c}
Total lipid (g/yolk)	3.4 ± 0.1	5.7 ± 0.1 ^{c}
Lipid class % (w/w) of total lipid		
TG	75.0 ± 0.8	70.5 ± 0.4 ^{b}
PL	18.5 ± 0.6	20.5 ± 0.8 ^{n}
CHOL	3.3 ± 0.5	6.6 ± 0.6 ^{b}
CE	2.1 ± 0.2	1.7 ± 0.1 ^{n}
FFA	1.2 ± 0.1	0.86 ± 0.07 ^{a}
mg fatty acid/yolk		
16:0	668.5 ± 30.0	1086.5 ± 27.3 ^{c}
18:0	242.7 ± 9.7	413.8 ± 12.0 ^{c}
18:1	966.2 ± 42.4	1757.7 ± 61.6 ^{c}
18:2	545.3 ± 20.4	689.6 ± 71.1 ^{a}
18:3	44.2 ± 2.1	54.1 ± 6.2 ^{n}
20:4	51.2 ± 1.1	73.1 ± 3.8 ^{b}
22:6	67.0 ± 2.7	93.8 ± 4.7 ^{a}

Figure 3.9: Embryo growth during the "normal" (○) and "small" (●) size egg experiments are shown. Mean ± S.E.M. of 4 sample replicates.

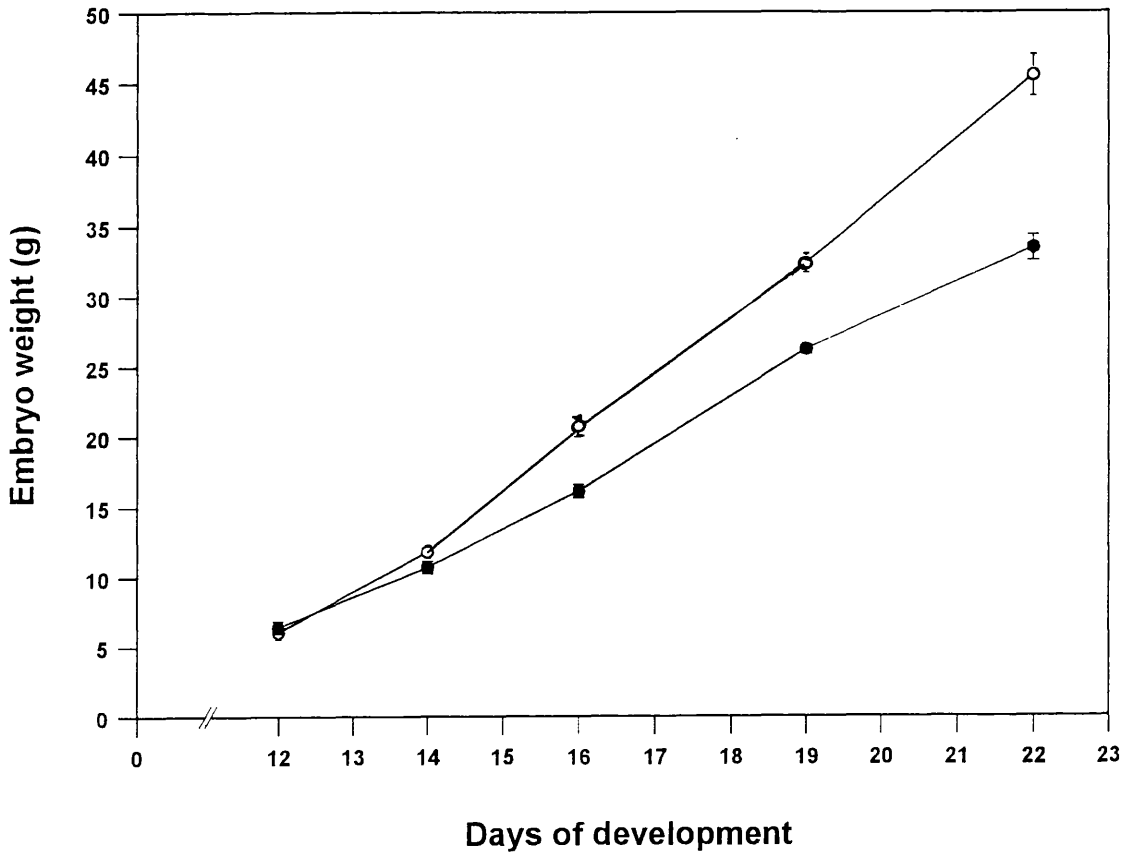

Embryo weight (g)
50
45
40
35
30
25
20
15
10
5
0
0
12
13
14
15
16
17
18
19
20
21
22
23
Days of development

Figure 3.10 : Adipose tissue development in the embryo. The adipose depots defined in the method section, were quantitatively dissected from the embryos. The TG content (mg/depot) of the adipose tissue are depicted for the "normal" size (○) and "small" (●) egg experiments. Means ± S.E.M. of 4 replicates. Comparison between days 22 and 19 $^{c}P<0.01$, $^{d}P<0.02$.

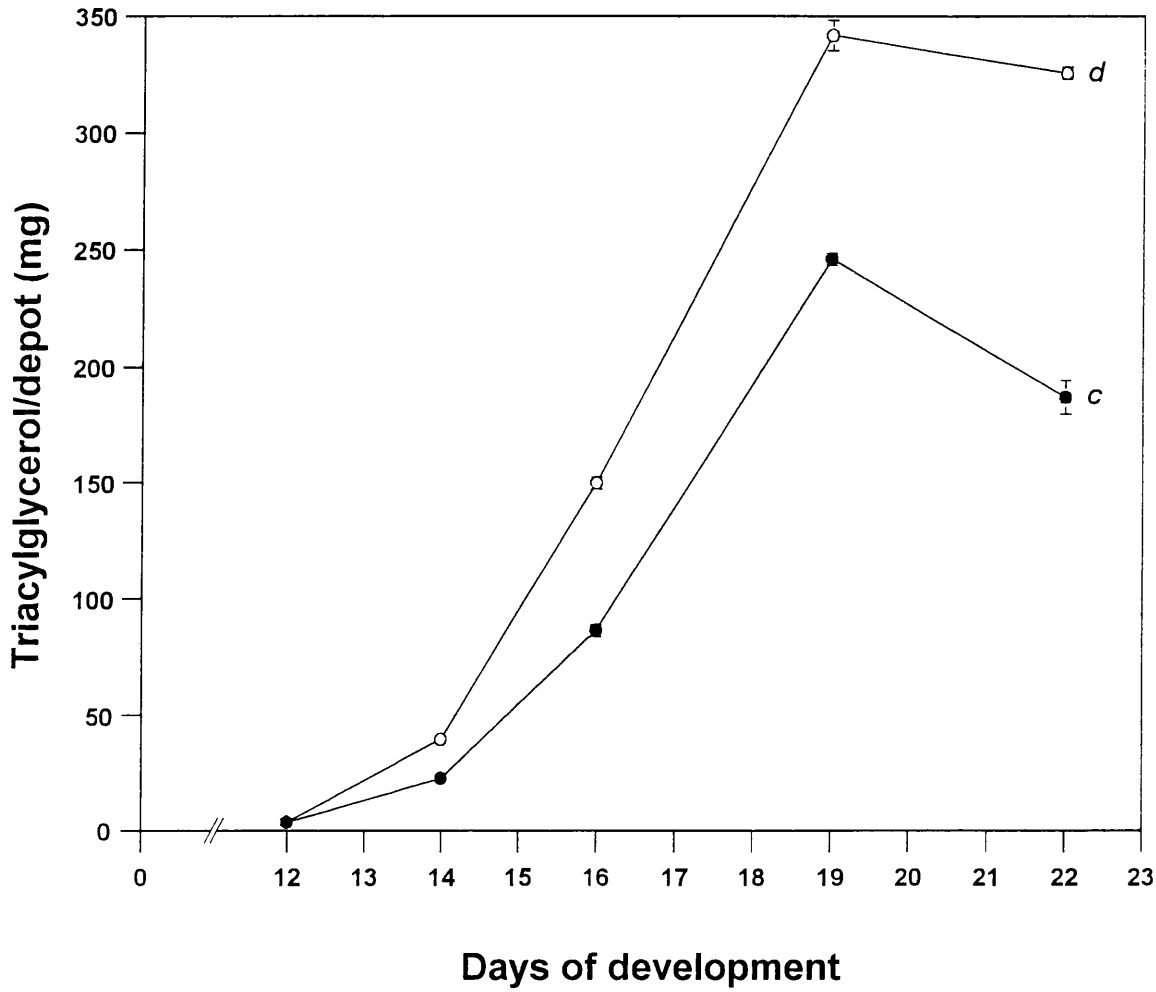

Triacylglycerol/depot (mg)
350
300
250
200
150
100
50
0
0
12
13
14
15
16
17
18
19
20
21
22
23
Days of development
d
c

Table 3.35: Triacylglycerol content as a percentage of whole embryo weight. Significant differences for value on the same line (between experiments) are illustrated with superscript character $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$, $^{n}P>0.05$ not significant.

Days of incubation	Age of parental flock 24	33
14	0.20	0.27^{c}
16	0.53	0.80^{c}
19	0.94	0.99^{n}
22	0.56	0.75^{c}

Figure 3.11: Dynamics of lipid deposition in adipose depot against lipid transfer from yolk during embryo development ("normal" size egg experiment). Linear regression (omitting the day 22 value), r^2= 0.997, y=0.13*x - 62.8.

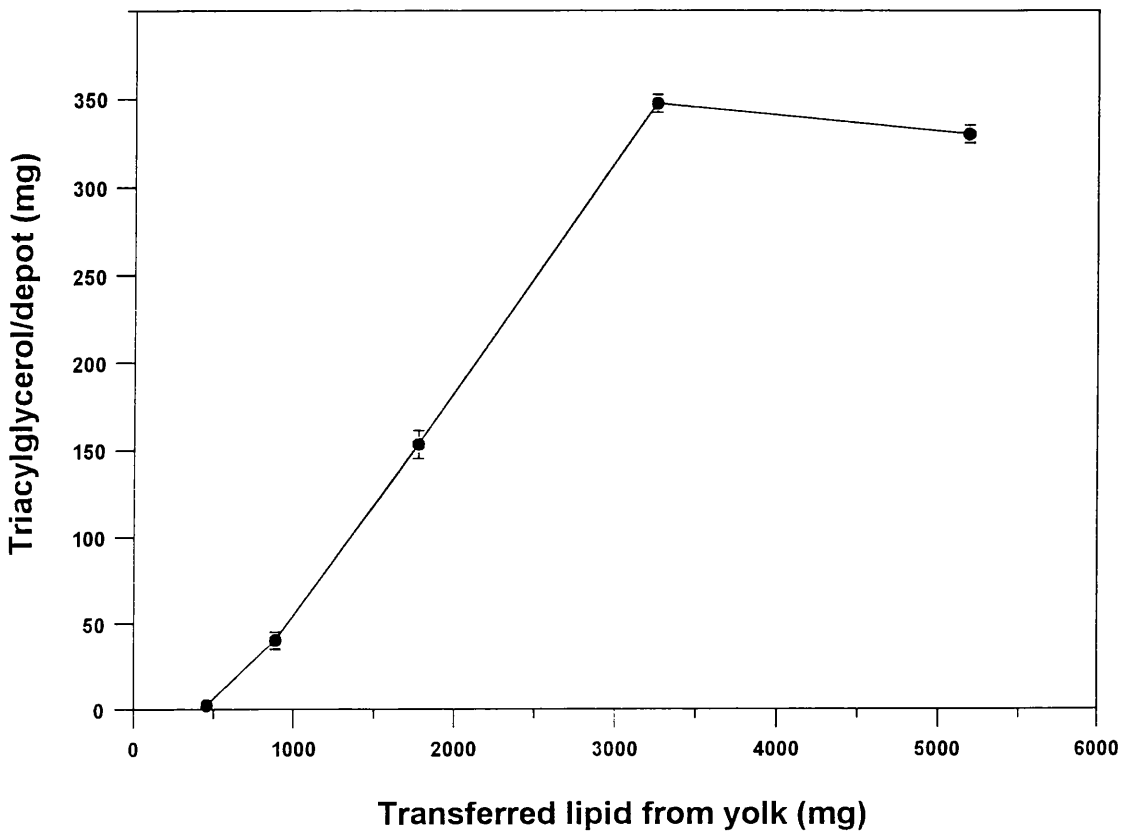

Triacylglycerol/depot (mg)
350
300
250
200
150
100
50
0
0
1000
2000
3000
4000
5000
6000
Transferred lipid from yolk (mg)

Table 3.36: Fatty acid composition of adipose tissue triacylglycerol during development ("normal" eggs).

Fatty acids (%)	Day 12	14	16	19	PH1
16:0	30.6 ± 0.5	28.8 ± 1.1	28.4 ± 0.2	28.6 ± 0.2	28.0 ± 0.3
16:1	2.5 ± 0.1	2.8 ± 0.1	2.9 ± 0.03	2.8 ± 0.03	2.8 ± 0.1
18:0	7.5 ± 0.3	7.8 ± 0.3	8.0 ± 0.7	8.0 ± 0.03	7.9 ± 0.1
18:1	33.0 ± 1.7	32.5 ± 1.1	35.5 ± 0.1	37.5 ± 0.1	40.6 ± 0.2
18:2	16.8 ± 1.3	16.2 ± 0.6	17.0 ± 0.02	17.9 ± 0.1	16.8 ± 0.02
18:3	0.9 ± 0.2	1.0 ± 0.2	1.3 ± 0.02	1.4 ± 0.04	1.3 ± 0.03
20:4	2.0 ± 0.1	4.2 ± 1.5	1.6 ± 0.01	1.2 ± 0.03	1.0 ± 0.2
20:5	0.3 ± 0.1	0.29 ± 0.02	0.2 ± 0.0	0.1 ± 0.01	0.07 ± 0.00
22:6	5.4 ± 0.2	4.84 ± 0.7	3.8 ± 0.02	1.6 ± 0.03	0.7 ± 0.04

Table 3.37: Fatty acid composition of adipose tissue triacylglycerol during development ("small" eggs).

Fatty acids (%)	Day 12	14	16	19	22
16:0	28.0 ± 0.2	27.0 ± 0.1	28.1 ± 0.2	28.4 ± 0.2	28.4 ± 1.3
16:1	2.5 ± 0.1	2.5 ± 0.02	2.8 ± 0.1	2.6 ± 0.0	1.8 ± 0.6
18:0	7.8 ± 01	7.7 ± 0.06	7.2 ± 0.1	7.2 ± 0.3	7.1 ± 0.7
18:1	28.3 ± 0.1	31.0 ± 0.5	32.6 ± 0.1	33.5 ± 0.2	34.9 ± 0.8
18:2	20.2 ± 0.3	20.2 ± 0.4	20.8 ± 0.1	22.7 ± 0.5	23.8 ± 0.6
18:3	1.5 ± 0.4	1.5 ± 0.04	1.6 ± 0.05	1.6 ± 0.1	1.6 ± 0.1
20:4	2.4 ± 0.1	2.2 ± 0.2	1.5 ± 0.0	1.2 ± 0.03	0.7 ± 0.1
20:5	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.04 ± 0.01
22:5	0.8 ± 0.0	0.7 ± 0.0	0.6 ± 0.1	0.4 ± 0.0	0.3 ± 0.0
22:6	7.4 ± 0.1	6.3 ± 0.1	4.0 ± 0.1	1.6 ± 0.0	0.6 ± 0.1

Figure 3.12: Oleic acid content of adipose triacylglycerol. The amounts (mg/depot) of oleic acid in adipose triacylglycerol are depicted for the "small" (○) and "normal" (●) size experiments. Means of 4 replicate tissue samples ± S.E.M.. Comparison between days 22 and 19 : $^{c}P<0.01$.

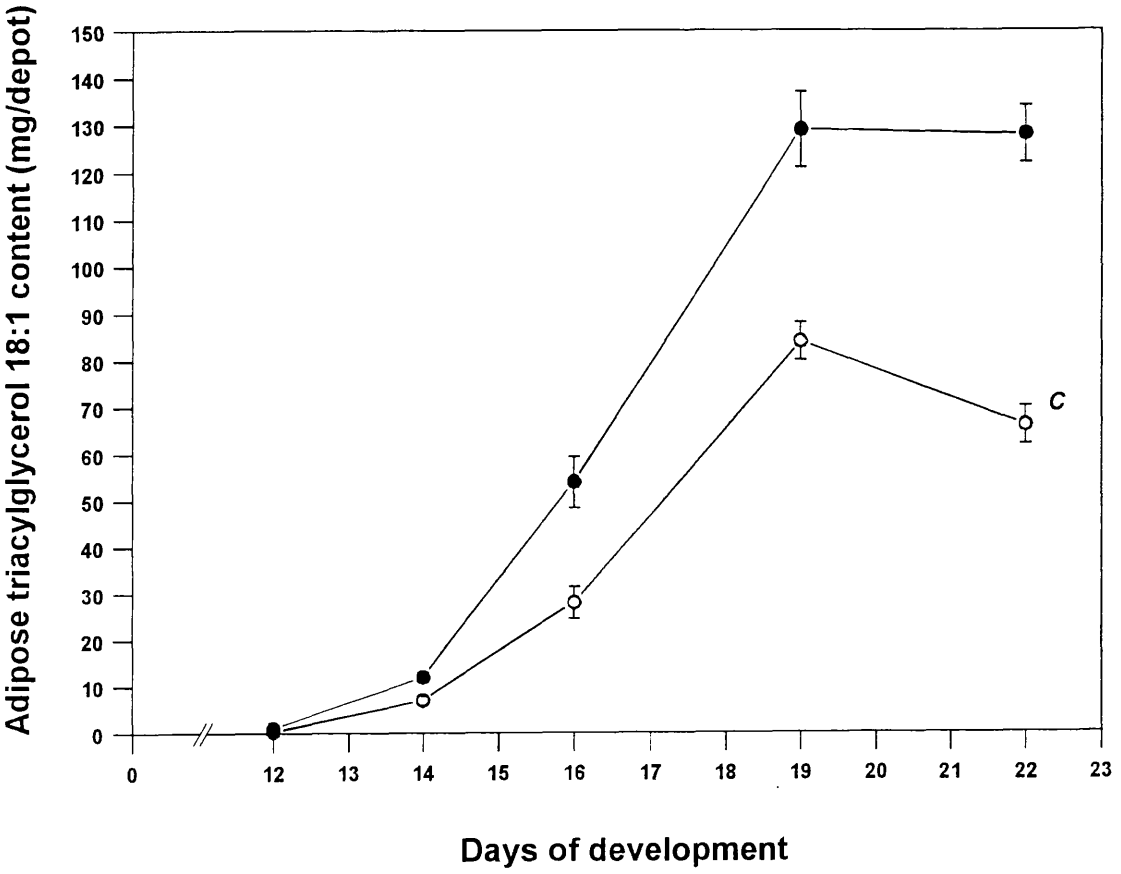

Adipose triacylglycerol 18:1 content (mg/depot)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
12
13
14
15
16
17
18
19
20
21
22
23
C
Days of development

Figure 3.13: DHA content of adipose triacylglycerol. The amounts (mg/depot) of DHA in adipose triacylglycerol are depicted for the "small" (○) and "normal" (●) size egg experiments. Means of 4 replicate tissue samples ± S.E.M.. Comparison between days 22 and 19 : $^{a}P<0.01$.

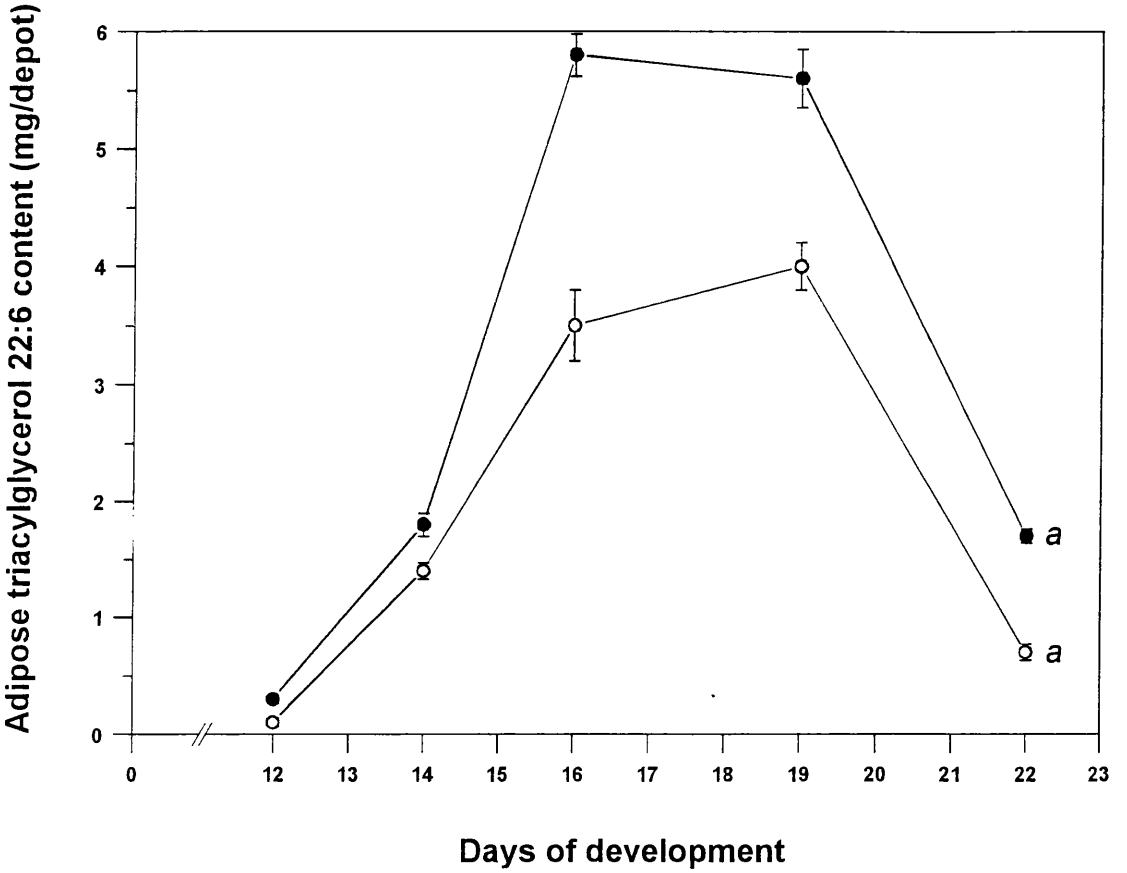
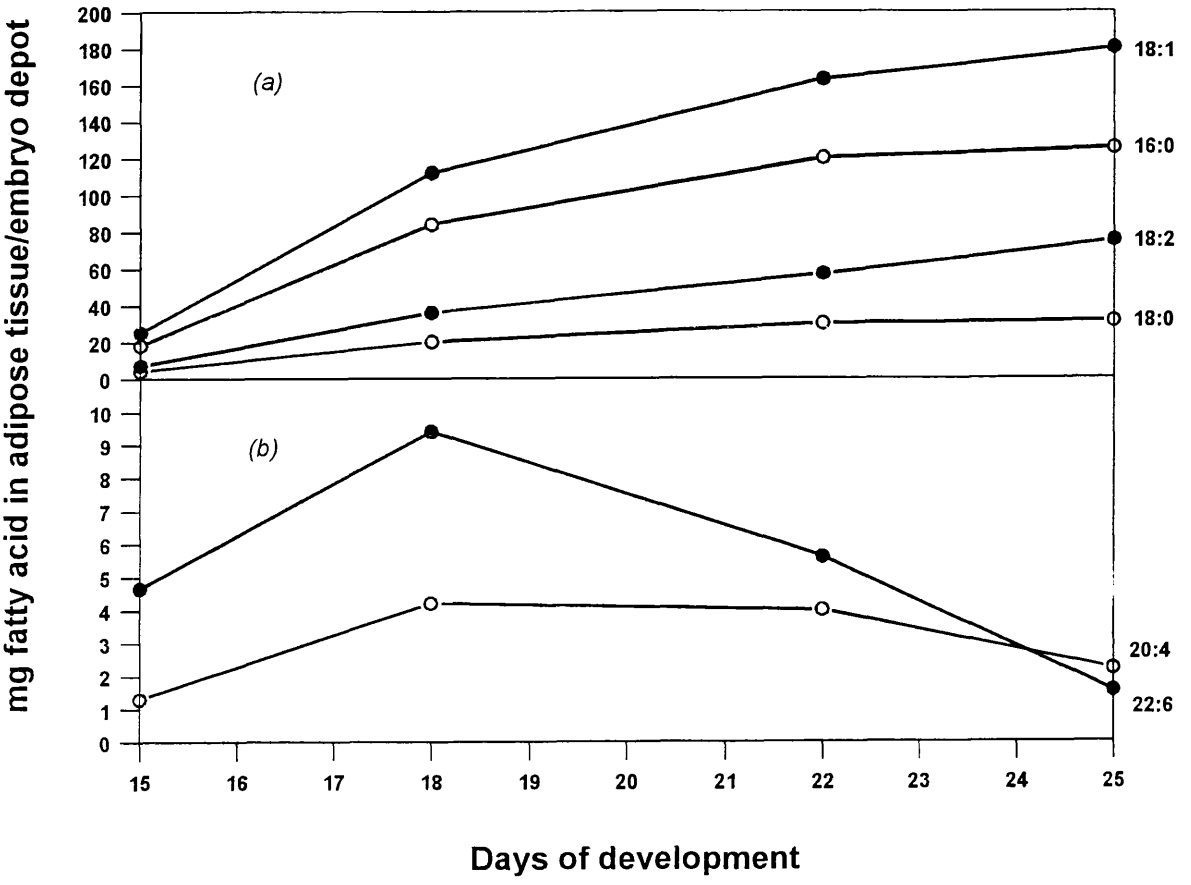

Adipose triacylglycerol 22:6 content (mg/depot)
6
5
4
3
2
1
0
0
12
13
14
15
16
17
18
19
20
21
22
23
Days of development
a
a

Table 3.38: Fatty acid composition (% w/w of total fatty acids) of adipose TG during development (experiment 3). Values are means ± S.E.M.. Each replicate refers to tissue from an individual embryo or chick. Days 22 and 25 are equivalent to 1 and 4 days respectively after hatching.

Fatty acids (%)	Day 15 (n=8)	18 (n=8)	22 (n=4)	25 (n=3)
mg TG/depot	66.0 ± 6.5	285.6 ± 23.5	402.3 ± 42.9	401.7 ± 48.0
Fatty acid (% w/w of total fatty acid)				
16:0	28.3 ± 0.3	29.5 ± 0.4	29.9 ± 0.5	27.8 ± 0.3
16:1	2.8 ± 0.1	3.5 ± 0.2	3.6 ± 0.3	3.9 ± 0.2
18:0	7.6 ± 0.3	7.4 ± 0.2	7.7 ± 0.3	7.1 ± 0.03
18:1	38.0 ± 0.4	39.7 ± 0.7	40.4 ± 0.7	41.3 ± 0.8
18:2	11.6 ± 0.4	13.1 ± 0.4	14.2 ± 0.5	16.6 ± 0.3
18:3	0.7 ± 0.04	0.7 ± 0.07	0.8 ± 0.0	1.5 ± 0.1
20:4	2.0 ± 0.2	1.5 ± 0.1	1.0 ± 0.04	0.5 ± 0.04
22:6	7.2 ± 0.2	3.4 ± 0.2	1.4 ± 0.2	0.3 ± 0.03

Figure 3.14: Major fatty acid content of adipose triacylglycerol. The amounts (mg/depot) of fatty acids in adipose triacylglycerol are depicted for experiment 3. Means of 4 replicate tissue samples and in all cases S.E.M. were less than 5% of the means.


mg fatty acid in adipose tissue/embryo depot
(a)
(b)
18:1
16:0
18:2
18:0
20:4
22:6
Days of development

3.1.6. Fatty acid composition of the lipids of the liver during development.

The fatty acid compositions of the liver were determined on the tissue samples from experiment 2 (defined as in Section *3.1.1*). In agreement with previous studies (Noble and Cocchi, 1990), the proportion of CE increased dramatically during development, comprising nearly 70% (w/w) of the total lipid by the time of hatching (Table 3.39). The changes in the amount of the lipid classes in the liver are illustrated in Figure 3.15. The proportion of DHA (% w/w of total fatty acids) in the TG fraction was very high (17.6%) at day 13, decreasing to about half this value just prior to hatching (Table 3.40). High levels (up to 24%) of AA were present in the PL fraction, which also contained significant levels of DHA (Table 3.41). The proportion of DHA in liver PL increased 1.5-fold over the hatching period. Cholesteryl oleate was the major component of the cholesteryl ester fraction throughout development (Table 3.42). These results are consistent with those of previous studies (Noble and Cocchi, 1990; section *1.3.5*). The FFA fraction also contained high levels of both AA and DHA (Table 3.43).

The absolute amounts (mg/liver) of the various fatty acids in the total lipids of the liver are shown in Figure 3.16 (a and b). 18:1 was the major component from day 14 onwards. The amounts of 20:4 and DHA in total liver lipid increased continuously until hatching and then decreased slightly by day 22.

Figure 3.17 illustrates the developmental changes in the absolute amounts (mg in lipid class per liver) of the main fatty acids in the different liver lipid classes. The major fatty acid, 18:1, mainly distributed into CE with lower amounts present in TG and PL (Figure 3.17 b), reflecting the massive deposition of cholesteryl oleate in the liver. A similar general pattern was observed for 18:2 (Figure 3.16), although the amount of this fatty acid present in CE was low compared to 18:1. In contrast, the saturated fatty acids, 16:0 and 18:0, were mainly distributed into PL, with lower amounts present in TG and CE (Figure 3.17 a). Almost all the 20:4 content of the liver was present as a component of PL (Figure 3.17 c). The amount of DHA as a component of PL increased continuously during embryonic development and continued to increase over the peri-hatching period. Whereas the amount of DHA in TG also increased up to day 19, the level then decreased by day 22 (Figure 3.17 d). Thus, during the hatching period, there was an increase in the DHA content of PL with a corresponding decrease in the amount of this fatty acid in TG. DHA was the only major fatty acid to exhibit an increase in the PL fraction at this time : the amounts of all the other major fatty acids in PL either decreased or remained constant between days 19 and 22 (Figure 3.17 a, b and c).

Table 3.39: Lipid compositions of the liver during development from experiment 2. Values are means ± S.E.M., n=4.

	Day 12	13	14	15	16	19	22
Liver fresh wt (mg)	79.0	134.7	238.0	330.4	459.7	654.7	510.0
Amount of total lipid (mg)	3.12	5.2 ± 0.2	13.3 ± 1.0	17.9 ± 0.4	25.8 ± 0.1	91.4 ± 0.6	75.1 ± 0.5
% total lipid							
PL	63.5 ± 1.0	57.7 ± 2.4	43.8 ± 1.8	40.2 ± 3.0	32.5 ± 3.1	13.2 ± 1.9	15.2 ± 1.2
CE	4.2 ± 0.4	11.2 ± 1.1	18.3 ± 1.7	28.7 ± 5.0	43.5 ± 3.2	69.0 ± 4.1	68.2 ± 4.8
TG	10.8 ± 0.5	8.8 ± 0.9	12.4 ± 0.8	11.0 ± 0.6	7 ± 0.8	5.0 ± 0.8	3.3 ± 0.7
CHOL	0.2 ± 0.02	7.3 ± 0.7	8.0 ± 0.9	8.3 ± 0.8	7.4 ± 0.3	5.5 ± 0.8	5.8 ± 0.1
FFA	13.8 ± 0.2	15 ± 1	15.5 ± 2.8	11.8 ± 0.8	9.6 ± 0.3	7.3 ± 0.7	7.5 ± 2.7

Figure 3.15: Content of lipids in the liver during development. CE (▲), FFA (○), PL (□), TG (●) and CHOL (Δ). Results are means of 4 replicates, S.E.M. were always less than 10 % of the means.

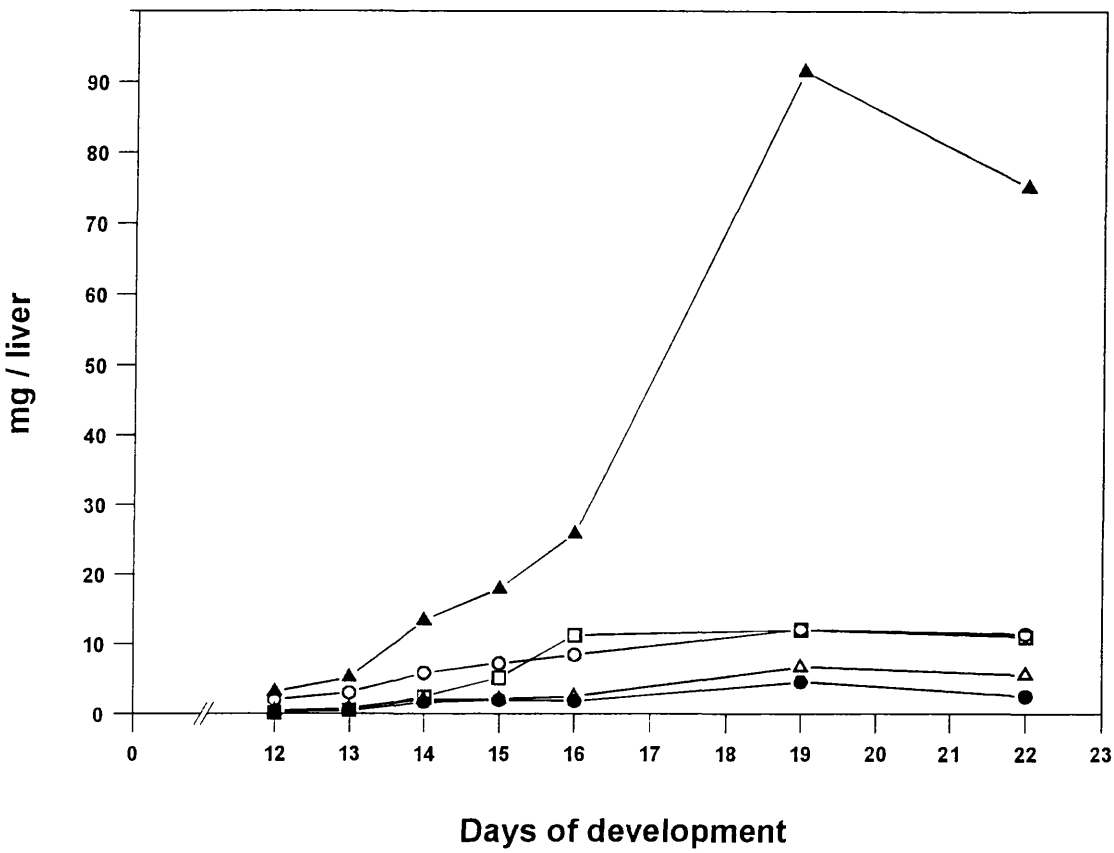

mg / liver
90
80
70
60
50
40
30
20
10
0
0
12
13
14
15
16
17
18
19
20
21
22
23
Days of development

Table 3.40: Fatty acid composition of liver triacylglycerol (% w/w total fatty acid). Means ± S.E.M., n=4.

% total fatty acid	Day 12	13	14	15	16	19	22
16:0	23.9 ± 1.5	26.4 ± 2.2	23.3 ± 2.1	26.6 ± 1.0	24.2 ± 1.7	20.8 ± 0.8	23.7 ± 7.9
18:0	9.6 ± 0.6	11.5 ± 1.5	8.9 ± 0.7	10.8 ± 0.5	11.0 ± 1.2	9.9 ± 1.0	13.4 ± 1.2
18:1	36.4 ± 2.1	28.6 ± 4.4	38.4 ± 4.1	32.3 ± 1.0	34.6 ± 5.8	44.7 ± 1.9	43.5 ± 4.0
18:2	10.7 ± 0.6	10.1 ± 1.5	13.7 ± 1.4	11.8 ± 0.5	12.7 ± 1.7	12.24±0.02	11.9 ± 4.0
18:3	0.60 ± 0.03	0.68 ± 0.11	1.0 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.85 ± 0.02	0.4 ± 0.4
20:4	3.9 ± 0.6	5.1 ± 0.9	3.1 ± 0.2	3.9 ± 0.2	4.0 ± 0.6	3.3 ± 0.2	2.4 ± 0.4
22:6	14.9 ± 0.3	17.6 ± 2.2	11.7 ± 0.7	13.8 ± 0.5	12.7 ± 1.2	8.2 ± 0.1	4.7 ± 1.2

Table 3.41: Fatty acid composition of liver phospholipid (% w/w total fatty acid). Means ± S.E.M., n=4.

% total fatty acid	Day 12	13	14	15	16	19	22
16:0	22.6 ± 1.1	22.1 ± 0.8	20.7 ± 1.8	20.6 ± 0.9	19.5 ± 2.1	18.5 ± 2.3	18.9 ± 1.8
18:0	22.8 ± 0.1	23.4 ± 1.3	24.3 ± 1.5	24.3 ± 1.2	24.7 ± 2.4	26.9 ± 3.2	26.1 ± 2.1
18:1	7.6 ± 0.0	7.1 ± 0.2	8.1 ± 0.7	8.0 ± 0.3	7.9 ± 0.8	8.4 ± 1.4	8.0 ± 0.5
18:2	8.9 ± 0.1	9.87 ± 0.42	13.0± 1.0	14.4 ± 0.7	15.9 ± 1.6	14.0 ± 2.4	13.0 ± 0.1
18:3	0.1 ± 0.0	0.10 ± 0.01	0.10 ± 0.03	0.08 ± 0.03	0.07 ± 0.04	0.09 ± 0.05	0.1 ± 0.1
20:4	23.2 ± 0.6	24.4 ± 1.2	23.6 ± 1.6	22.3 ± 0.9	22.2 ± 2.2	21.9 ± 3.6	18.5 ± 1.0
22:6	14.7 ± 0.1	13.0 ± 0.8	10.3 ± 0.7	10.3 ± 0.5	9.7 ± 0.9	10.3 ± 1.8	15.4 ± 3.2

Table 3.42: Fatty acid composition of liver cholesterol ester (% total fatty acid). Means ± S.E.M., n=4.

% total fatty acid	Day 12	13	14	15	16	19	22
16:0	10.3 ± 0.4	5.7 ± 0.7	4.3 ± 0.2	4.1 ± 1.0	2.9 ± 0.2	2.8 ± 0.2	2.7 ± 0.3
18:0	5.5 ± 0.0	3.1 ± 0.3	3.3 ± 0.2	3.2 ± 0.6	3.0 ± 0.2	3.3 ± 0.3	3.3 ± 0.4
18:1	74.2 ± 8.7	66.4 ± 6.9	77.5 ± 7.1	73.6 ± 13.3	76.9 ± 5.3	74.3 ± 5.4	77.3 ± 7.0
18:2	7.5 ± 1.2	11.9 ± 1.2	11.3 ± 1.4	12.6 ± 2.5	12.4 ± 1.0	13.8 ± 1.0	12.5 ± 0.3
18:3	0.4 ± 0.4	0.52 ± 0.09	0.47 ± 0.09	0.67 ± 0.10	0.78 ± 0.05	0.81 ± 0.06	0.7 ± 0.0
20:4	0.1 ± 0.1	2.7 ± 0.3	0.7 ± 0.6	1.2 ± 0.2	1.0 ± 0.1	1.8 ± 0.1	1.2 ± 0.1
22:6	2.0 ± 1.6	9.7 ± 0.9	2.4 ± 2.1	4.6 ± 1.1	3.0 ± 0.5	3.1 ± 0.4	2.2 ± 0.0

Table 3.43: Fatty acid composition of liver free fatty acids (% total fatty acid). Means ± S.E.M., n=4.

% total fatty acid	Day 12	13	14	15	16	19	22
16:0	55.8 ± 19.5	19.0 ± 0.7	17.9 ± 1.7	20.6 ± 1.6	17.6 ± 0.6	14.7 ± 2.3	14.8 ± 4.4
18:0	3.9 ± 10.4	12.1 ± 0.9	11.7 ± 1.7	11.2 ± 1.2	11.7 ± 0.5	13.8 ± 1.3	12.0 ± 4.2
18:1	0.7 ± 2.8	18.2 ± 1.0	21.0 ± 2.7	21.1 ± 1.9	24.3 ± 0.6	23.3 ± 2.5	28.1 ± 8.9
18:2	3.5 ± 0.5	11.7± 0.6	13.6 ± 1.9	14.6 ± 1.2	15.9 ± 0.4	13.7 ± 1.4	14.8 ± 5.9
18:3	1.5 ± 0.1	0.36 ± 0.01	0.48 ± 0.05	0.37 ± 0.09	0.59 ± 0.02	0.43 ± 0.06	0.7 ± 0.4
20:4	27.6 ± 5.1	22.3 ± 1.5	18.5 ± 3.2	16.5 ± 1.5	14.4 ± 0.3	17.4 ± 1.6	11.6 ± 3.9
22:6	7.0 ± 3.2	16.3 ± 0.7	16.8 ± 3.0	15.5 ± 1.6	15.5 ± 0.5	16.6 ± 1.5	17.9 ± 8.1

Figure 3.16: Amount (mg/liver) of individual major fatty acids. (a) 16:0 and 18:0 (○), 18:1 (□), 18:2 (■),(b) AA (●) and DHA (Δ). Results are means of 4 replicates, S.E.M. were always less than 10 % of the means.

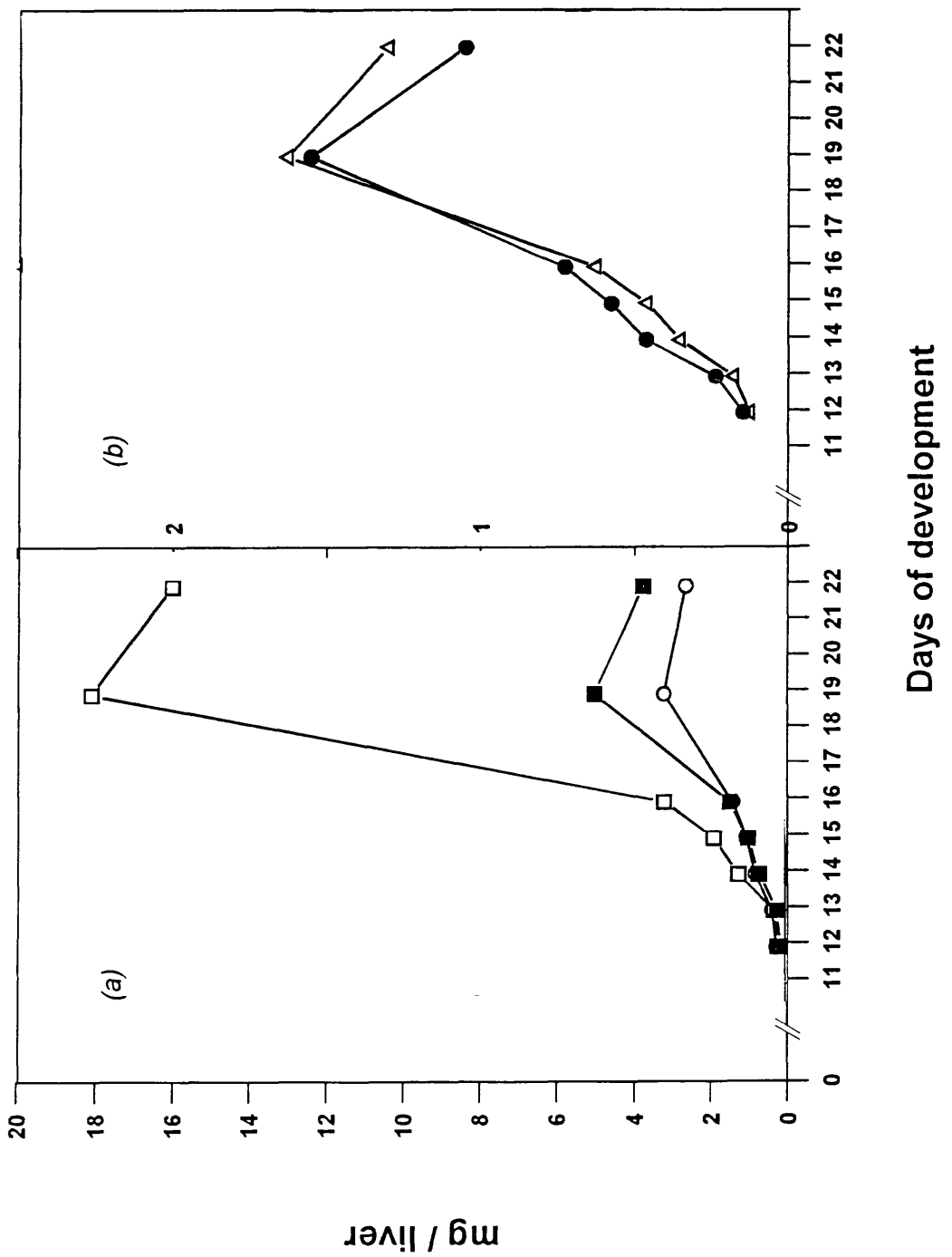
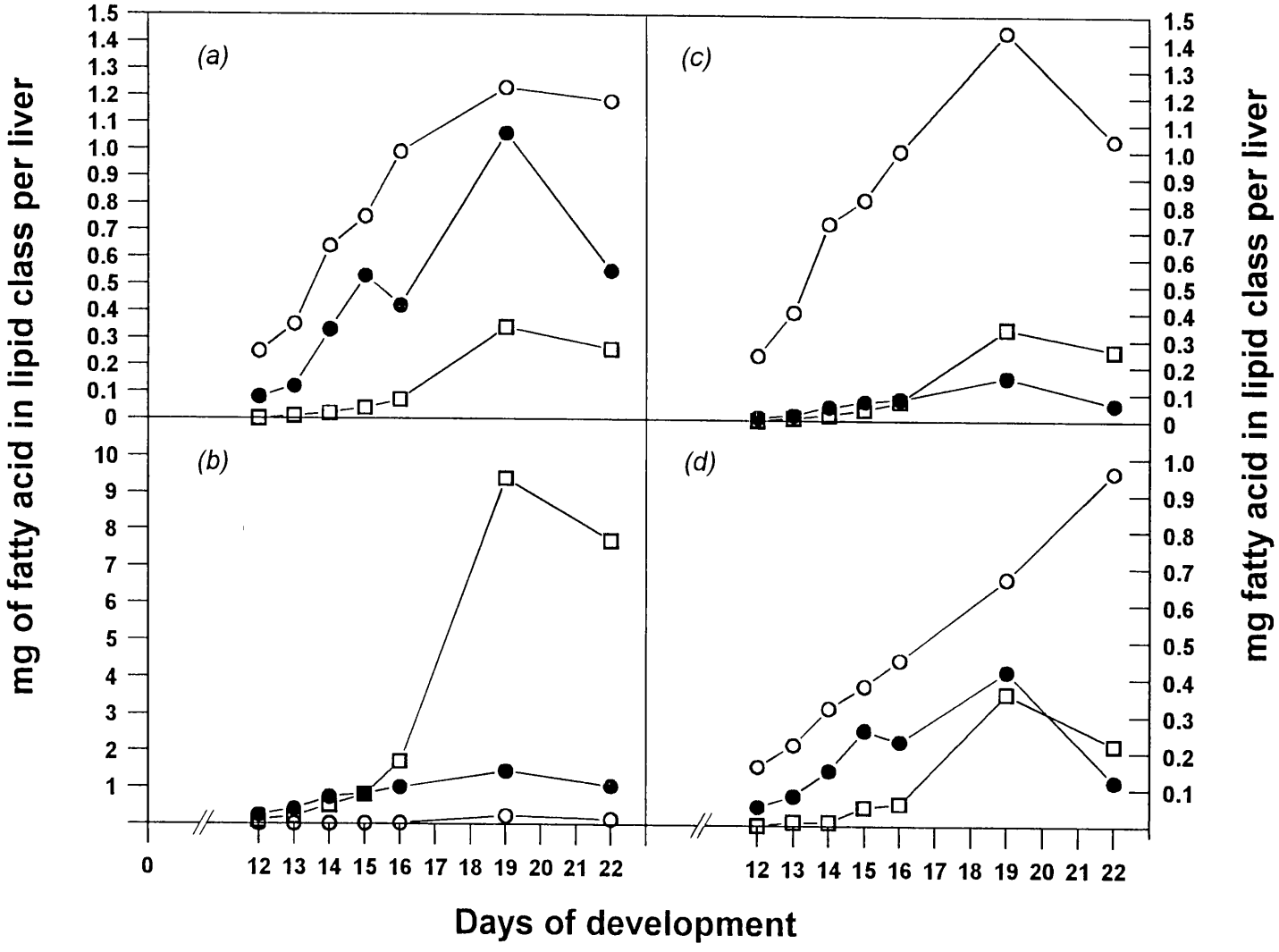

mg / liver
Days of development
(a)
(b)
0
2
4
6
8
10
12
14
16
18
20
1
11 12 13 14 15 16 17 18 19 20 21 22

Figure 3.17: Distribution of major fatty acids between lipid classes in the liver. The amounts (mg of fatty acid in lipid class per liver) of (a) 16:0, (b) 18:1, (c) AA and (d) DHA , determined in the PL (○), TG (●) and CE (□). Results are means of 4 replicates, S.E.M. were always less than 10 % of the means.


(a)
(b)
(c)
(d)
mg of fatty acid in lipid class per liver
mg fatty acid in lipid class per liver
Days of development
0
12 13 14 15 16 17 18 19 20 21 22

3.1.7. Fatty acid composition of the lipids of the heart during development

The fatty acid compositions of the lipid classes were determined in hearts obtained from experiment 2 (defined in section *3.1.1*). Unlike liver and adipose tissue, the heart contained relatively low levels of lipid, in which the structural lipid, PL, predominated. However, the amount of TG in the heart increased considerably by the peri-hatching period (Table 3.44). The TG fraction of heart exhibited certain similarities to that of adipose tissue, containing high levels of DHA and significant levels of AA at day 14, with decreased proportions of both these fatty acids at the later stages (Table 3.45). Although cholesteryl oleate was the major species of CE present in heart, the proportion of this species was considerably lower than in liver (Table 3.46). AA was a major component of the PL fraction (Table 3.47). The absolute amount (μg in lipid class/heart) of 18:1, AA and DHA in the PL and TG fractions are shown in Figure 3.18. The amount of 18:1 in both lipid classes increased throughout the period studied (Figure 3.18 a). The amount of AA in PL also increased continuously, but the level of this fatty acid in TG remained at a very low level throughout development (Figure 3.18 b). The results in Figure 3.18 c suggest that the maximum amount of DHA in both PL and TG had already been attained by day 14, with little change thereafter. It is clear from these results that the heart is not a major site of DHA accumulation.

3.1.8. Fatty acid composition of brain PL during development.

In order to investigate the effects of variations in the initial yolk lipid and DHA content on the subsequent composition of brain phospholipid, 3 experiments were performed using eggs of different sizes. These experiments correspond to experiments 1 and 2 described in section *3.1.1* (Table 3.1) and to another "small" egg experiment (experiment 4*)* performed for brain collection (Table 3.48). The relevant parameters of the eggs used for these experiments are given in Table 3.48. The major lipid classes present were PL and CHOL. Only trace levels of TG, CE and FFA were detected and these are not included in Tables 3.49, 3.50 and 3.51. The relative proportions of PL and CHOL did not change dramatically during development, although some increases in the proportion of CHOL were observed at the later stages, possibly reflecting myelination. The percentage composition of the PL fatty acids was also relatively constant during development. Within each experiment the proportion of DHA (% w/w of PL fatty acids) did not change to any extent from about day 12 to hatching, and composed up to 21% (w/w) of the total fatty acids.

The absolute amounts (mg/brain) of 16:0, AA and DHA in the brain PL are presented in Figure 3.19. The amounts (mg/brain) of DHA increased continuously throughout the developmental period studied approximately in parallel with the amounts of the other major fatty acids, and largely reflecting the increase in the amount of PL and the growth of the brain. Only a small proportion (less than 10%; Table 3.48) of the DHA initially present in the yolk was recovered in the brain phospholipids of the newly hatched chick. In spite of the much lower initial DHA content of the yolk lipid in experiment 4 compared to experiment 2, there was only a minor difference in the neonatal brain DHA content between the 2 experiments. However, the extremely low initial yolk DHA level in experiment 1 was reflected in a greater reduction in the amount of this fatty acid in the neonatal brain (Table 3.48).

Table 3.44: Lipid composition of heart during development. Means ± S.E.M., n=4.

	Day 14	15	16	19	22
Heart fresh weights (g)	0.11	0.14	0.16	0.23	0.29
mg total lipid/heart	3.0 ± 0.1	3.9	4.7 ± 0.06	6.6 ± 0.07	8.7 ± 0.3
% total lipid					
TG	7.2 ± 0.4	7.7	6.5 ± 0.3	15.4 ± 3.3	20.7 ± 1.8
PL	67.4 ± 0.6	63.0	63.8 ± 1.1	57.5 ± 2.3	60.5 ± 1.3
CE	4.3 ± 0.1	6.2	6.8 ± 0.3	6.8 ± 0.3	6.2 ± 0.7
FFA	8.5 ± 0.4	10.2	10.3 ± 1.1	8.0 ± 1.0	6.6 ± 0.2
CHOL	12.6 ± 0.4	12.9	12.4 ± 0.2	11.7 ± 0.5	6.3 ± 0.7

Table 3.45: Fatty acid composition (% w/w total fatty acids) of heart triacylglycerol. Means ± S.E.M., n=4.

acid	Day 14	15	16	19	22
16:0	30.3 ± 0.6	29.2 ± 2.5	33.5 ± 0.3	30.3 ± 1.1	31.3 ± 0.9
18:0	9.7 ± 0.1	7.8 ± 1.1	8.7 ± 0.2	6.8 ± 0.6	6.4 ± 0.3
18:1	28.7 ± 0.8	34.8 ± 2.9	30.1 ± 0.3	35.4 ± 2.0	41.5 ± 0.5
18:2	13.3 ± 0.2	17.5 ± 2.6	14.3 ± 0.2	17.4 ± 0.8	17.2 ± 0.5
18:3	1.2 ± 0.02	1.1 ± 0.2	1.2 ± 0.05	0.9 ± 0.1	0.7 ± 0.05
20:4	5.1 ± 0.4	3.4 ± 1.2	5.0 ± 0.2	2.6 ± 0.6	1.1 ± 0.09
22:6	9.2 ± 1.0	3.3 ± 1.7	5.3 ± 0.2	1.7 ± 0.2	0.7 ± 0.07

Table 3.46: Fatty acid composition (% w/w total fatty acids) of heart cholesteryl ester. Means ± S.E.M., n=4.

acid	Day 14	16	19	22
16:0	12.2 ± 0.2	10.3 ± 0.2	8.8 ± 0.7	10.5 ± 0.2
18:0	4.7 ± 0.2	4.6 ± 0.2	4.4 ± 0.2	3.9 ± 0.1
18:1	47.2 ± 0.2	51.6 ± 0.7	52.9 ± 1.2	50.6 ± 2.3
18:2	21.7 ± 0.3	21.7 ± 0.4	21.6 ± 0.5	22.4 ± 1.3
18:3	1.0 ± 0.02	1.0 ± 0.04	1.1 ± 0.07	0.7 ± 0.07
20:4	5.2 ± 0.1	4.3 ± 0.3	5.0 ± 0.3	5.8 ± 0.8
22:6	4.7 ± 0.3	3.6 ± 0.05	4.1 ± 0.1	2.8 ± 0.2

Table 3.47: Fatty acid composition (% w/w total fatty acids) of heart phospholipid. Means ± S.E.M., n=4.

acid	Day 14	16	19	22
16:0	27.2±0.2	28.0±0.6	26.4±0.6	24.2±0.5
18:0	20.2±0.6	18.9±0.4	19.9±0.1	20.7±0.2
18:1	17.1±0.4	17.6±0.5	16.7±0.5	16.1±0.3
18:2	8.8±0.2	10.6±0.2	10.1±0.3	11.0±0.3
18:3	ND	ND	ND	ND
20:4	17.8±0.3	18.9±0.9	23.2±0.3	24.5±0.5
22:6	7.3±0.7	4.3±0.3	3.7±0.6	2.5±0.3

Table 3.48: Relevant lipid parameters for experiments 1, 2 and 4.

	Experiments		
	1	2	4
Initial yolk lipid content (g)	3.8	5.6	3.4
Initial yolk lipid DHA content (mg)	33	94	67
One day post hatch brain DHA (mg)	2.8	3.6	3.4
% DHA recovered in the brain (compared to yolk)	8.5	3.8	5.1

Figure 3.18: Absolute amounts of 18:1 (a), AA (b) and DHA (c) in the chick embryo heart. Amounts (μg in lipid class / heart) of these fatty acids in TG (●) and PL (○).

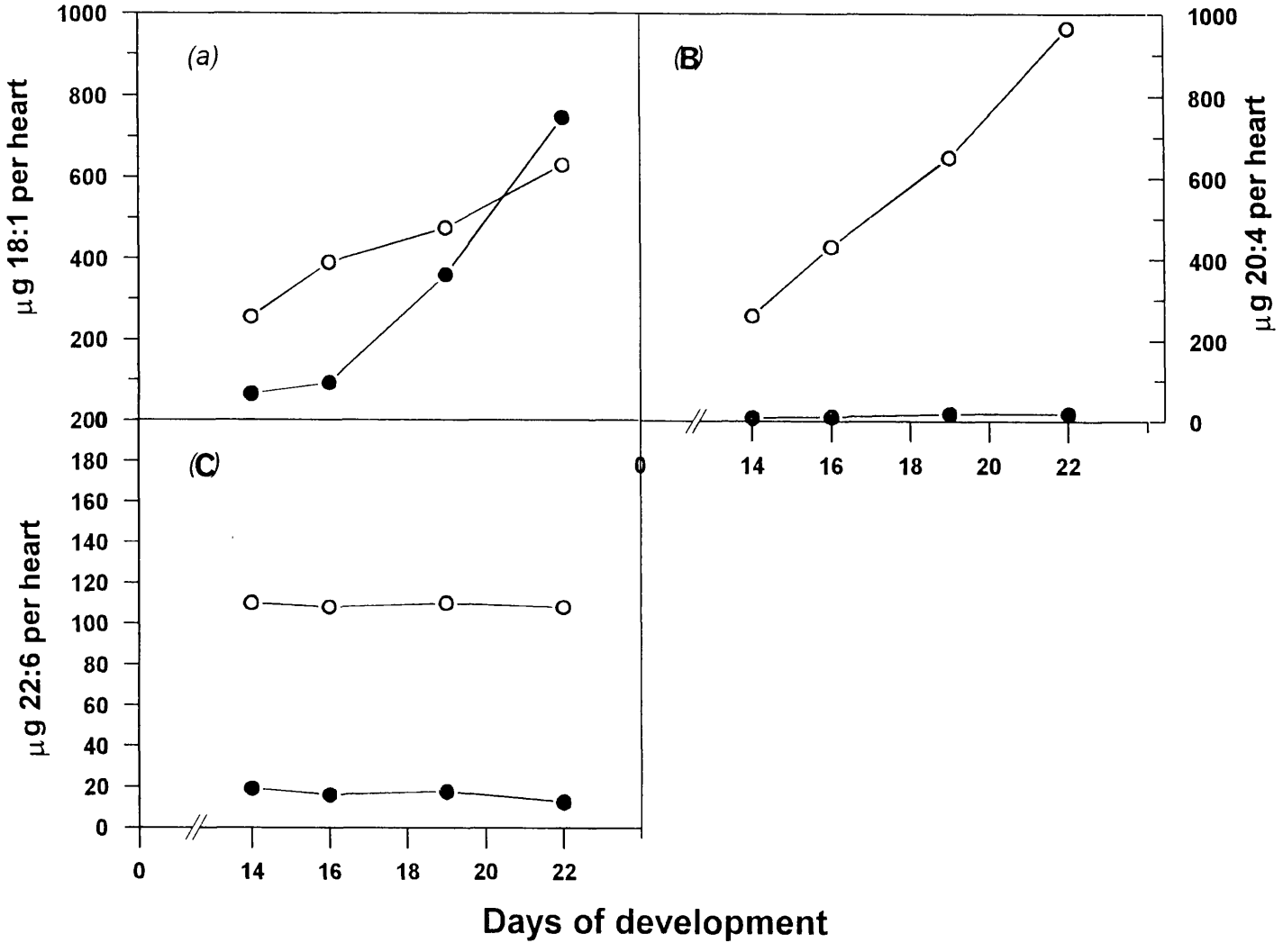

(a)
(B)
(C)
µg 18:1 per heart
µg 20:4 per heart
µg 22:6 per heart
Days of development
1000
800
600
400
200
180
160
140
120
100
80
60
40
20
0
14
16
18
20
22

Table 3.49: Brain lipid composition (experiment 1). Statistical comparisons were made against the D11 value, differences at $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$, $^{n}P>0.05$ not significant.

	Day 11	13	15	18	22	25
Fresh brain weight (g)	0.175 ± 0.004	0.296 ± 0.005	0 42 ± 0.03	0.72 ± 0.02	0.90 ± 0.04	1.07 ± 0.09
Total lipid content (mg/brain)	4.0 ± 0.1	6.4 ± 0.2	10.6 ± 0.3	17.6 ± 1.5	32.1 ± 1.8	47.7 ± 4.5
Lipid classes (% w/w of total lipid)						
PL	76.4± 1.6	73.3± 0.5	74.5± 2.1	73.3± 0.8^{n}	64.5± 1.1^{c}	64.1± 4.7^{a}
CHOL	22.3± 2.2	26.7± 0.5	25.5± 2.1	26.7± 0.8^{n}	35.5± 1.1^{c}	35.9± 4.7^{a}
Fatty acid composition (% w/w of total fatty acids) of brain PL						
16:0	37.0 ± 0.6	36.7 ± 0.9	34.8 ± 1.6	34.8 ± 2.5	29.3 ± 1.3	27.3±2.4^{n}
18:0	14.5 ± 0.2	29.8 ± 0.6	17.8 ± 1.0	17.5 ± 1.1	18.9 ± 1.3	19.8±1.9^{n}
18:1	18.1 ± 0.2	17.6 ± 0.3	17.4 ± 0.9	17.4 ± 1.2	19.5 ± 1.3	20.7±2.4^{n}
18:2	2.1 ± 0.1	2.1 ± 0.09	2.4 ± 0.13	2.0 ± 0.05	2.3 ± 0.07	2.1±0.3^{n}
18:3	0.3 ± 0.05	0.3 ± 0.01	0.3 ± 0.02	0.4 ± 0.04	0.7 ± 0.07	0.8±0.1^{n}
20:4	10.1 ± 0.2	10.1 ±0.02	9.8 ± 0.5	8.7 ± 2.5	11.2 ± 0.4	10.8±1.4^{n}
22:6	17.9 ± 0.3	17.9 ± 0.3	19.4 ± 0.9	19.2 ± 1.1	18.2 ± 1.3	18.4±2.4^{n}

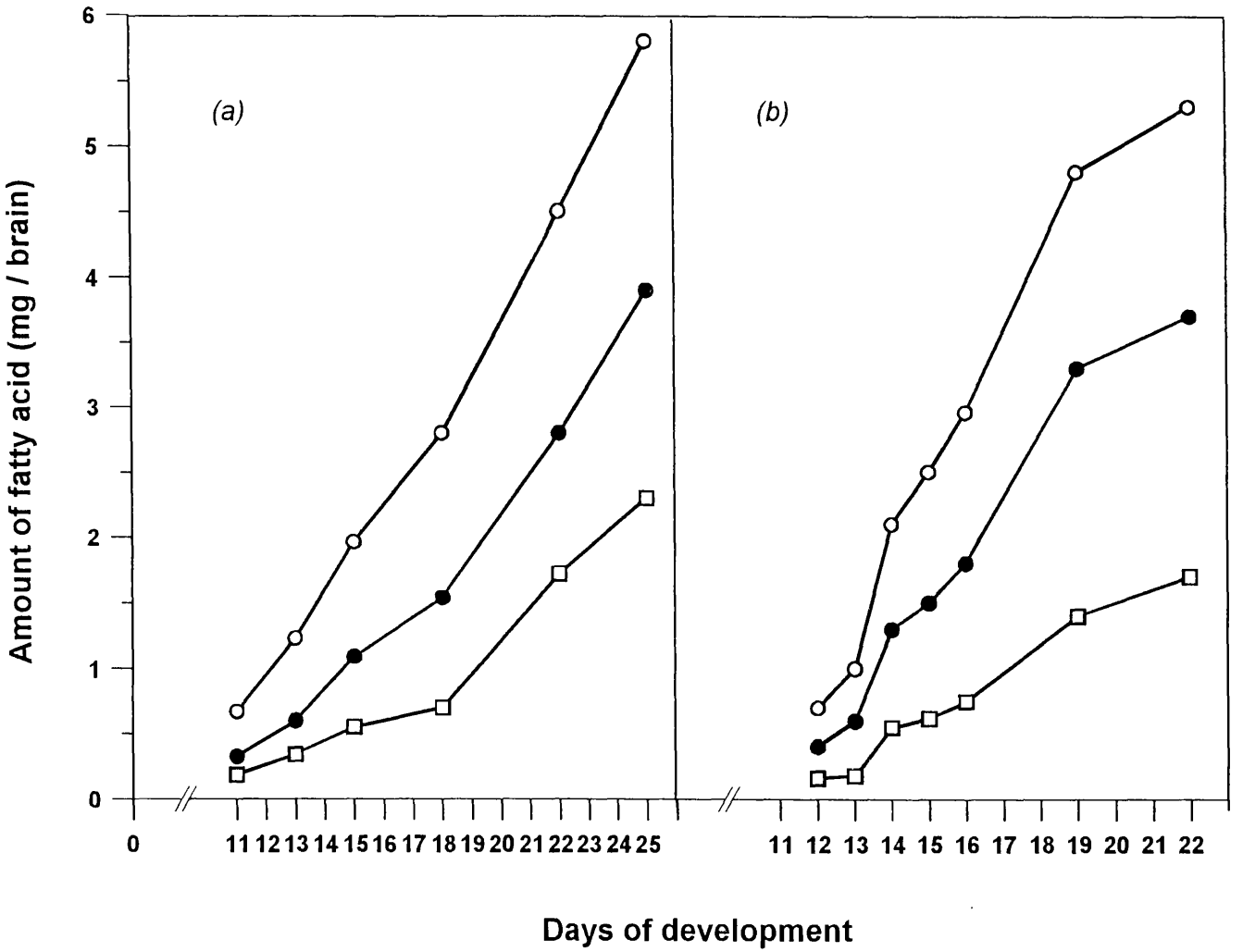
Table 3.50: Lipid composition of the brain during development (experiment 2).

	Day 12	13	14	15	16	19	22
Fresh brain weight (g)	0.142	0.321	0.559	0.559	0.691	1.033	1.022
Total lipid content (mg/brain)	3.79 ± 0.02	8.0 ± 1.0	13.1 ± 1.0	14.8 ± 0.5	19.1 ± 0.7	34.5 ± 1.8	43.0 ± 2.0
Fatty acid composition (% w/w of total fatty acids) of brain PL							
16:0	36.7 ± 1.6	36.4 ± 3.6	33.8 ± 1.1	34.7 ± 1.2	34.3 ± 0.6	31.7 ± 1.5	29.7 ± 1.7
18:0	15.7 ± 0.5	14.6 ± 1.4	16.1 ± 0.5	15.3 ± 0.6	16.2 ± 0.3	17.8 ± 0.7	18.5 ± 1.1
18:1	15.7 ± 0.5	18.2 ± 1.8	17.7 ± 0.6	18.1 ± 0.8	17.4 ± 0.1	17.2 ± 0.66	18.5 ± 1.1
18:2	2.1 ± 0.05	2.2 ± 0.4	0.2 ± 0.08	2.2 ± 0.1	2.3 ± 0.0	1.8 ± 0.3	2.2 ± 0.3
18:3	0.3 ± 0.01	0.3 ± 0.02	0.3 ± 0.01	0.3 ± 0.003	0.3 ± 0.01	0.5± 0.02	0.7 ± 0.005
20:4	8.4 ± 0.3	6.6 ± 2.2	8.8 ± 0.3	8.6 ± 0.3	8.7 ± 0.2	9.2 ± 0.3	9.5 ± 0.6
22:6	21.0 ± 1.0	21.8 ± 2.2	20.9 ± 0.6	20.8 ± 0.7	20.8 ± 0.6	21.8 ± 0.7	20.8 ± 1.7

Table 3.51: Lipid composition of the brain during development (experiment 4).

	Day 12	14	16	19	22
Fresh brain weight (g)	0.27	0.38	0.48	0.69	0.76
Total lipid content (mg/brain)	6.6 ± 0.2	10.1 ± 0.2	13.3 ± 0.4	25.2±0.6	35.9 ± 0.5
Lipid classes (% w/w of total lipid)					
PL	78.2 ± 0.2	77.7 ± 0.6	77.8 ± 0.3	76.7 ± 0.7	75.2 ± 0.2
CHOL	21.9 ± 0.2	22.3 ± 0.6	22.2 ± 0.3	23.3 ± 0.7	24.8 ± 0.2
Fatty acid composition (% w/w of total fatty acids) of brain PL					
16:0	37.0 ± 0.2	36.3 ± 0.4	36.5 ± 0.2	34.4 ± 0.2	32.1 ± 0.2
16:1	3.3 ± 0.03	3.5 ± 0.04	3.1 ± 0.1	2.4 ± 0.03	1.6 ± 0.07
18:0	12.6 ± 0.1	12.9 ± 0.1	13.1 ± 0.1	15.9 ± 0.1	17.4 ± 0.1
18:1	16.4 ± 0.2	16.7 ± 0.3	15.8 ± 0.1	16.2 ± 0.3	16.4 ± 0.2
18:2	2.3 ± 0.1	2.3 ± 0.07	2.2 ± 0.07	2.0 ± 0.06	2.1 ± 0.1
18:3	0.2 ± 0.05	0.2 ± 0.0	0.1 ± 0.0	0.4 ± 0.0	0.5 ± 0.0
20:4	7.8 ± 0.1	8.1 ± 0.3	7.8 ± 0.1	8.1 ± 0.3	9.2 ± 0.2
20:5	2.0 ± 0.0	1.9 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	2.3 ± 0.1
22:6	16.7 ± 0.4	17.0 ± 0.2	17.5 ± 0.2	17.8 ± 0.2	17.2 ± 0.2

Figure 3.19: Absolute amounts of 16:0, AA, DHA in the brain PL. Amounts (mg/brain) of 16:0 (○), AA (□) and DHA (•) are represented for experiments 1 (*a*) and 2 (*b*).


(a)
(b)
Amount of fatty acid (mg / brain)
Days of development
0
1
2
3
4
5
6
0
11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
11 12 13 14 15 16 17 18 19 20 21 22

3.2-BRAIN PHOSPHOLIPID CLASSES AND THEIR FATTY ACID COMPOSITION

3.2.1. Isolation of PL classes by HPLC

A typical chromatogram illustrating the separation of the major PL classes isolated from the brain of the day-old chick, using the HPLC system described in Section 2.2.2, is shown in Figure 3.20. It can be seen that the method allows PE, PI, PS and PC to be clearly resolved. Three criteria were used to identify the peaks: (i) The sequence of elution and retention times were compared with the published values in the original paper by Christie (1985) in which this method was first described. (ii) Comparison with the retention times of purchased standards of PE, PI, PS and PC on this HPLC system. (iii) Collection of the peaks and analysis by HPTLC as described in section *2.2.4.*. Identification of the spots on the plate was by comparison with the original paper by Olsen and Henderson (1989) and by co-chromatography of standards.

3.2.2. Fatty acid composition of PL classes during brain development in the chicken

Brain samples collected during experiment 4 were analysed. PC was the major PL fraction present, followed by PE, PS and PI. The proportions of the PL classes in the brain did not change significantly during embryonic development, although the proportion of PE decreased by one day after hatching (Table 3.52).

Each PL class exhibited a highly characteristic fatty acid composition: PC consisted largely of saturated and mono-unsaturated acids, mainly 16:0 and 18:1 with only very low levels of polyunsaturates including AA and DHA (Table 3.53). This profile was fairly constant during development apart from an increase in the proportion of 18:0 and a decrease in that of DHA. The composition of PE was very different from that of PC. DHA was the major component of PE (up to 40% w/w of total fatty acid) throughout embryonic development and high levels of AA (approximately 13%) were also present (Table 3.54). 18:0 was the major saturated fatty acid in PE. The fatty acid profile of PE was relatively constant throughout embryogenesis, but the proportion of DHA decreased significantly by the first day after hatching, with a corresponding increase in the 18:0 and 18:1 levels. The composition of PS (Table 3.55) exhibited many similarities to that of PE. Again, DHA was the major fatty acid at days 12 and 14 (up to 40% w/w), and the major saturated component was 18:0. The level of AA was lower in PS than in PE. The fatty acid profile of PS did however change somewhat

during development; the proportion of DHA decreased by approximately 50% by hatching, while the proportions of 18:0 and 18:1 increased significantly.

The composition of PI (Table 3.56) was characterised by very high levels (up to 45 % w/w) of AA, but only low levels of DHA. 18:0 was the major saturated component, and relatively high levels of 18:1 were also present. The highest levels of AA were found at the earlier stages (days 12 and 14), decreasing significantly by the time of hatching, with corresponding increases in the level of 18:1 and also 18:2.

In conclusion, these results indicate that the DHA content of the chick embryo brain is present mainly in PE, with significant levels also present in PS. AA is present largely in PE and PI, whereas only relatively low amounts of the long chain polyunsaturates are present in PC.

3.2.3. Fatty acid composition of PL classes in the brain of the duck embryo

Lipid analysis of the initial egg yolk (i.e. prior to incubation) were carried out and results are given in Table 3.57. The general characteristics were similar between the two species. DHA was present in lower amounts in the duck yolk compared to the chicken's (nearly 6-fold).

Samples were collected from duck embryos at days 18 and 21 of the 28 day embryonic period. As in the chick, the major PL class in duck brain was PC, followed by PE, PS and PI at day 18. However, an increased proportion of PI was evident at day 21 (Table 3.58).

There was a general similarity in the fatty acid composition of the PL classes between the two species. PC from duck brain consisted mainly of saturated and monounsaturated fatty acids, particularly 16:0 and 18:1 with only low level of DHA and AA (Table 3.59). PE was rich in both DHA and AA (Table 3.59); however the ratio of DHA / AA was much lower in the duck than in the chicken, reflecting the low levels of DHA and high level of AA in the lipids of the initial yolk of the duck eggs (Table 3.57). PS ,in the duck, was also rich in DHA (Table 3.60), but again the levels of this fatty acid were lower than in the chicken. PI was rich in AA, but the proportion of DHA increased considerably between days 18 and 20 (Table 3.60).

Figure 3.20: Brain PL separation. Chromatogram obtained by HPLC. RT: retention time, FC: free cholesterol, PE:phosphatidylethanolamine, PI: phosphatidylinositol, PS: phosphatidylserine, PC: phosphatidylcholine and SPH: sphingomyelin.

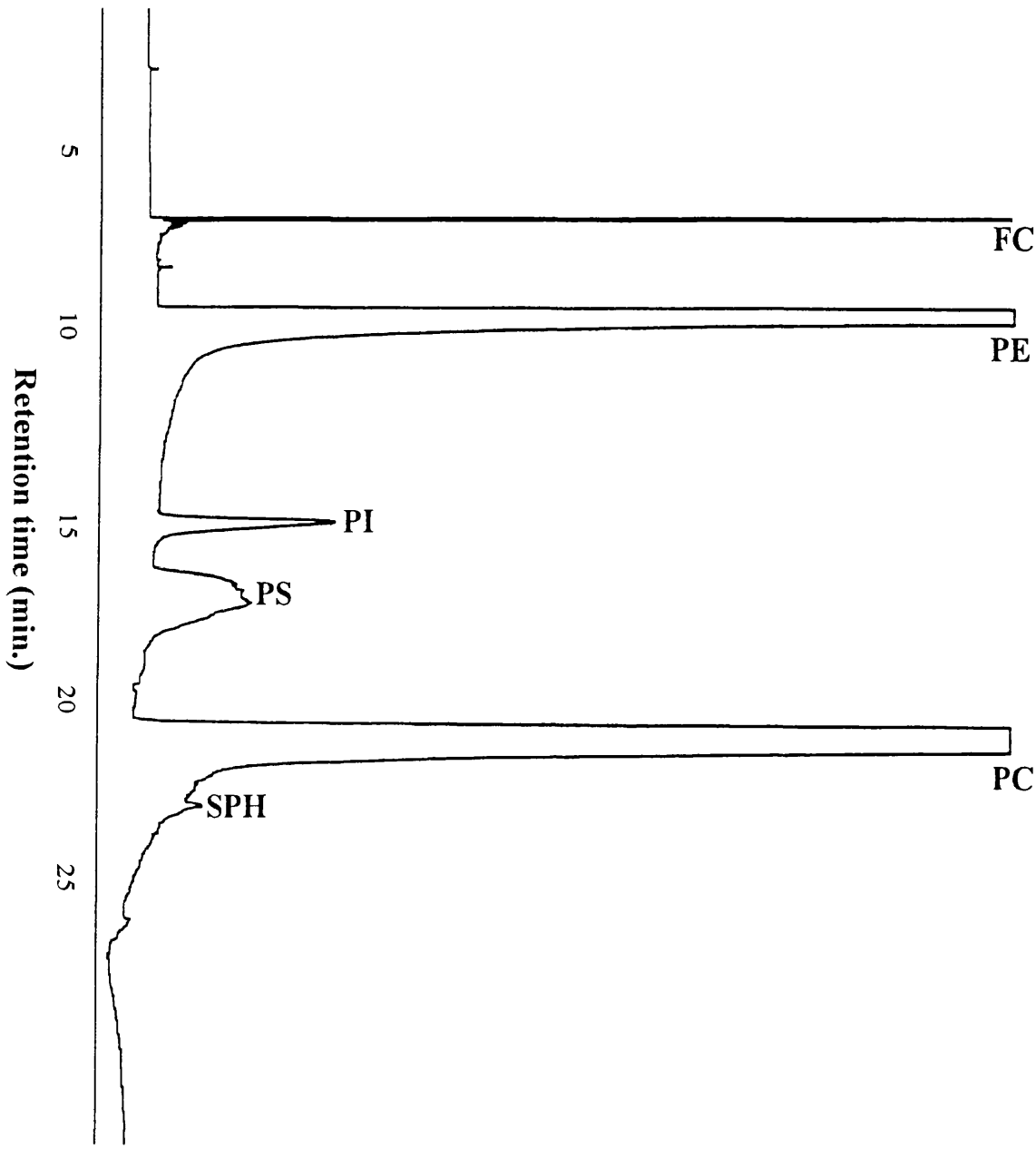

Retention time (min.)
5
10
15
20
25
FC
PE
PI
PS
PC
SPH

Table 3.52: Proportions of the major phospholipid classes of the brain during chick embryo development. Results are the % (w/w) of total PL, and refer to means ± S.E., n=4. Statistical comparisons against the D12 value are indicated, differences at $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$, $^{n}P>0.05$ not significant.

	Day 12	14	16	19	22
PC	43.8 ± 1.7	40.8 ± 0.6^{n}	45.3 ± 2.1^{n}	42.6 ± 1.2^{n}	48.5 ± 5.5^{n}
PE	28.5 ± 1.2	34.6 ± 4.5^{n}	30.3 ± 1.1^{n}	27.5 ± 0.6^{n}	19.5 ± 1.0^{b}
PS	18.3 ± 0.8	19.5 ± 3.6^{n}	17.5 ± 2.5^{n}	18.5 ± 2.0^{n}	19.2 ± 4.6^{a}
PI	9.4 ± 0.9	15.4 ± 2.7^{n}	6.9 ± 1.3^{n}	11.4 ± 1.1^{n}	12.8 ± 0.1^{n}

Table 3.53: Fatty acid composition of phosphatidylcholine (% w/w of total fatty acids). Values are means ± S.E., n=4. Statistical comparisons against the D12 value are indicated, differences at $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$, $^{n}P>0.05$ not significant.

	Day 12	14	16	19	22
C16:0	59.1 ± 2.1	61.4 ± 1.1	59.3 ± 1.0	57.2 ± 2.4^{n}	56.2 ± 1.6^{n}
C16:1	2.2 ± 1.3	ND	2.0 ± 1.2	ND	ND
C18:0	4.8 ± 0.1	3.8 ± 0.8	4.8 ± 0.3	7.9 ± 0.8^{b}	10.7 ± 0.9^{b}
C18:1	22.7 ± 0.2	23.8 ± 0.2	22.9 ± 0.5	24.7 ± 1.5^{n}	25.2 ± 0.1^{c}
C18:2	2.6 ± 0.1	2.6 ± 0.1	2.8 ± 0.1	2.2 ± 0.1^{c}	2.58 ± 0.01^{n}
C18:3	0.25 ± 0.02	0.18 ± 0.03	0.20 ± 0.01	0.3 ± 0.1^{n}	0.6 ± 0.1^{a}
C20:4	2.9 ± 0.2	3.0 ± 0.1	2.6 ± 0.3	3.3 ± 0.3^{n}	2.1 ± 0.2^{n}
C22:6	3.5 ± 0.4	4.1 ± 0.2	4.2 ± 0.2	4.0 ± 0.2^{n}	1.7 ± 0.5^{a}

Table 3.54: Fatty acid composition of phosphatidylethanolamine (% w/w of total fatty acids). Values are means ± S.E., n=4. Statistical comparisons against the D12 value are indicated, differences at $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$, $^{n}P>0.05$ not significant.

	D12	14	16	19	22
C16:0	14.5 ± 0.7	13.5 ± 1.2	15.4 ± 1.6	12.6 ± 0.8	10.0 ± 0.2^{b}
C18:0	20.0 ± 1.0	21.4 ± 0.5	22.3 ± 0.5	25.3 ± 1.5	31.1 ± 1.7^{c}
C18:1	10.2 ± 0.6	9.0 ± 0.4	9.2 ± 0.1	11.7 ± 2.1	18.9 ± 0.6^{a}
C18:2	0.89 ± 0.02	0.87 ± 0.04	1.20 ± 0.04	0.7 ± 0.2	1.5 ± 0.1^{b}
C18:3	0.31 ± 0.01	0.23 ± 0.01	0.26 ± 0.01	0.3 ± 0.1	1.3 ± 0.1^{c}
C20:4	12.7 ± 0.1	13.0 ± 0.4	12.5 ± 0.4	13.7 ± 0.8	14.2 ± 0.8^{n}
C22:6	39.0 ± 1.4	40.5 ± 0.9	37.5 ± 1.5	35.4 ± 2.0	22.7 ± 0.4^{c}

Table 3.55: Fatty acid composition of phosphatidylserine (% w/w of total fatty acids). Values are means ± S.E., n=4. Statistical comparisons against the D12 value are indicated, differences at $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$, $^{n}P>0.05$ not significant.

	Day 12	14	16	19	22
C16:0	8.9 ± 0.5	10.1 ± 0.5	13.9 ± 1.5	24.2 ± 5.5	9.2 ± 0.2^{n}
C16:1	0.3 ± 0.3	0.7 ± 0.7	0.2 ± 0.2	10.4 ± 0.9	ND
C18:0	35.8 ± 1.0	37.4 ± 1.0	37.3 ± 0.6	25.9 ± 0.4	47.9 ± 1.6^{c}
C18:1	8.7 ± 0.8	7.0 ± 0.3	7.9 ± 0.6	13.7 ± 1.7	17.45 ± 0.03^{c}
C18:2	1.9 ± 0.3	1.4 ± 0.3	1.5 ± 0.3	2.0 ± 0.5	2.28 ± 0.03^{n}
C18:3	0.29 ± 0.03	0.17 ± 0.01	0.17 ± 0.06	0.43 ± 0.01	1.15 ± 0.02^{c}
C20:4	3.1 ± 0.2	3.2 ± 0.2	4.1 ± 0.4	2.1 ± 0.8	2.73 ± 0.03^{n}
C22:6	39.4 ± 0.9	39.7 ± 0.6	33.9 ± 1.0	18.1 ± 4.6	18.7 ± 1.7^{a}

Table 3.56: Fatty acid composition of phosphatidylinositol (% w/w of total fatty acids). Values are means ± S.E., n=4. Statistical comparisons against the D12 value are indicated, differences at $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$, $^{n}P>0.05$ not significant.

	Day 12	14	16	19	22
C16:0	6.2 ± 0.7	3.5 ± 0.5	10.2 ± 1.8	9.4 ± 1.8	7.8 ± 0.1^{n}
C18:0	36.3 ± 0.3	37.6 ± 0.9	35.3 ± 6.7	37.9 ± 1.4	38.4 ± 1.3^{n}
C18:1	12.0 ± 0.6	9.0 ± 0.9	19.8 ± 3.3	11.7 ± 0.9	19.0 ± 0.4^{c}
C18:2	0.8 ± 0.1	0.6 ± 0.2	1.7 ± 0.7	1.0 ± 0.2	5.6 ± 0.4^{c}
C18:3	ND	ND	0.1 ± 0.1	ND	0.9 ± 0.3
C20:4	40.5 ± 0.3	45.0 ± 0.6	26.1 ± 9.0	35.2 ± 2.4	23.3 ± 0.6^{c}
C22:6	3.10 ± 0.6	3.8 ± 0.6	6.4 ± 0.9	4.0 ± 1.1	3.0 ± 0.7^{n}

Table 3.57: Characteristics of duck and chicken eggs prior to incubation. Values are the means ± S.E.M. of measurements on 4 eggs with the exception of egg weights where n=10.

	Duck	Chicken
Egg weight (g)	78.9 ± 1.0	62.2 ± 0.7
Total lipid (g/yolk)	6.8 ± 0.1	5.7 ± 0.1
Lipid class % (w/w) of total lipid		
TG	70.8 ± 0.5	70.5 ± 0.4
PL	21.7 ± 0.3	20.5 ± 0.8
CHOL	4.9 ± 0.2	6.6 ± 0.6
CE	0.8 ± 0.2	1.7 ± 0.1
FFA	0.8 ± 01	0.86 ± 0.07
mg fatty acid/yolk		
16:0	2122 ± 34.0	1086.5 ± 27.3
18:0	234.0 ± 10.9	413.8 ± 12.0
18:1	3534.0 ± 77.6	1757.7 ± 61.6
18:2	395 ± 21.0	689.6 ± 71.1
18:3	121 ± 4.9	54.1 ± 6.2
20:4	105 ± 7.0	73.1 ± 3.8
22:6	16.0 ± 1.0	93.8 ± 4.7

Table 3.58: Proportion of the major phospholipid classes of the brain during duck embryo development (% w/w of total phospholipid). Means ± S.E., n=4.

	PC	PE	PS	PI
Day 18	59.1 ± 1.4	19.1 ± 1.8	12.1 ± 0.5	9.7 ± 0.5
Day 21	48.8 ± 1.6	29.8 ± 1.3	6.3 ± 1.5	15.1 ± 1.4

Table 3.59: Fatty acid composition of phosphatidylcholine (PC) and phosphatidylethanolamine for duck brain (PE; % w/w of total fatty acids). Means ± S.E., n=4, when statistical differences between days 18 and 21: values differing at $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

PC	18	21	PE	Day 18	21
16:0	57.4 ± 0.4	60.90 ± 1.2[a]	16:0	24.0 ± 3.3	13.9 ± 1.5[a]
18:0	4.69 ± 0.04	4.8 ± 0.4	18:0	21.5 ± 2.6	20.5 ± 2.3
18:1	30.5 ± 0.4	27.3 ± 0.8[b]	18:1	18.4 ± 0.8	15.2 ± 0.7[a]
18:2	1.2 ± 0.1	0.8 ± 0.1[a]	18:2	0.5 ± 0.5	0.3 ± 0.2
18:3	0.33 ± 0.02	0.40 ± 0.02[a]	18:3	0.5 ± 0.1	0.49 ± 0.03
20:4	3.4 ± 0.2	3.2 ± 0.3	20:4	18.8 ± 1.1	20.7 ± 1.6
22:5	ND	ND	22:5	1.62 ± 0.02	1.9 ± 0.1[a]
22:6	1.9 ± 0.2	1.9 ± 0.2	22:6	16.1 ± 3.4	25.5 ± 0.5[a]

Table 3.60: Fatty acid composition of phosphatidylserine (PS) and phosphatidylinositol for duck brain (PI; % w/w of total fatty acids). Means ± S.E., n=4, when statistical differences between days 18 and 21: values differing at $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

PS	Day 18	21	PI	Day 18	21
16:0	3.5 ± 0.3	13.9 ± 3.3[a]	16:0	3.9 ± 0.4	4.9 ± 1.1[c]
18:0	45.9 ± 1.5	42.3 ± 1.8	18:0	30.2 ± 1.2	40.8 ± 0.4[c]
18:1	12.9 ± 1.3	13.1 ± 1.2	18:1	17.8 ± 2.4	13.3 ± 0.9
18:2	1.2 ± 0.5	0.56 ± 0.03	18:2	1.5 ± 0.3	ND[b]
18:3	0.41 ± 0.02	0.4 ± 0.1	18:3	0.33 ± 0.04	0.40 ± 0.04
20:4	7.4 ± 0.9	5.6 ± 0.2	20:4	36.7 ± 2.0	23.6 ± 1.1[b]
22:5	1.2 ± 0.3	0.9 ± 0.2	22:5	ND	0.74 ± 0.07[c]
22:6	26.9 ± 1.8	22.3 ± 1.6	22:6	3.0 ± 1.0	15.7 ± 0.3[c]

3.3 RELEASE OF FATTY ACIDS FROM ADIPOSE TISSUE *IN VITRO.*

The results in section *3.1.5* indicate firstly that the adipose tissue triacylglycerol (TG) represents a major store of DHA in the embryo and secondly that DHA appears to be selectively mobilised from the tissue *in vivo*. The studies described in the present section were designed to test the theory of preferential mobilisation of DHA by using *in vitro* methods. The basis of this approach was to maintain pieces of adipose tissue in culture medium for various periods of time and to compare the pattern of the free fatty acids (FFA) released into the culture medium with the fatty acid composition of the tissue TG. Three experiments (A, B and C), using different batches of chicken eggs, were designed to study different aspects of the release of fatty acids from the adipose tissue, including the effect of the incubation period and the effect of hormonal stimulation on the lipid release. An additional experiment was also carried out using adipose tissue from pheasant embryos.

Eggs were incubated as described in *2.1.1*. Adipose depots were collected and incubated as described in sections *2.1.2* and *2.3.1* respectively.

3.3.1. Preliminary experiment

A preliminary experiment (Experiment A) was carried out using adipose tissue from day 16 of the chicken's embryonic development. Adipose tissue pieces were incubated for 1 hour. The results (Table 3.61) showed that the free fatty acid profile of the incubation medium was significantly different from that of the initial adipose triacylglycerol (i.e. before incubation). The proportions of long chain PUFA (18:3, 20:4, 20:5 and 22:6) were significantly increased in the medium. The proportions of DHA and AA increased in the medium by 5- and 2.5-fold respectively.

3.3.2. Effect of different incubation period on the lipid and fatty acid release

In Experiment B, pieces of adipose tissue from chicken embryo were incubated for 1 or 24 hours. The amounts of total fatty acid released, expressed as a percentage of that initially present in the tissue TG, were compared for the two time periods. For both incubation times, a similar trend of fatty acid release was observed, the highest percentage rate of release being for day 14 of development followed by a decrease by day 18 and stabilisation over the hatching period (Figure 3.21). The lipid release was not linear with time with the percentage release by 24h being only 3-, 8- and 5-fold greater than after 1h for the samples at days 14, 18 and 22 respectively.

Tables 3.62, 3.63, 3.64 and 3.65 show the various fatty acid profiles for days 14, 16, 18 and 22 of development respectively. In each case the fatty acid profiles of the tissues did not significantly change before and after 1 hour of incubation (data not shown). Also no significant differences were found either at days 14 and 22 between fatty acid profiles of the tissue before and after 24 hours of incubation (data not shown). However at day 18, the proportion of DHA in the tissue TG did decrease significantly after 24h incubation. The ratio of the proportion of a particular fatty acid in the medium to its proportion in tissue TG is given in each case. A ratio superior to 1 indicates a preferential release of the fatty acid into the medium while a ratio inferior to 1 indicates a preferential retention of the fatty acid in the adipose tissue TG. As noticed in experiment A (section *3.3.1*) long chain PUFA (18:3, 20:4, 20:5, 22:5 and 22:6) were preferentially released in the medium for both the 1 and the 24 hour incubations. All monounsaturated and saturated fatty acids with the exception of 18:0 were preferentially retained in the tissue TG. For days 16 and 18 of development, the percentages of 20:4, 22:5 and 22:6 in the 24 hour incubation medium were lower than the values in the 1 hour medium. Figure 3.22 shows the logarithmic values of the ratios from Tables 3.62, 3.63, 3.64 and 3.65. Logs were used in order to express the ratios superior and inferior to 1 on the same scale. A $\log_{10}$ value equal to zero would indicate that the percentage of that fatty acid released into the medium was identical to that in the tissue whereas a positive value indicates preferential release and a negative value indicates preferential retention.

3.3.3.Effect of glucagon on the extent of lipolysis and on the profile of fatty acid released

Two concentrations of glucagon were used in experiment C: 100 ng/ml and 1 µg/ml of medium. Adipose tissues pieces from chicken embryo were incubated for 1 hour. An analysis of variance was carried out on the data using Minitab version 8.2. The variables analysed were the percentage of tissue lipid mobilised and the ratios of % fatty acid in medium FFA/% in adipose TG taking into consideration the day of development, the treatments and the interaction of both of these factors. Figure 3.23 shows the percentage of tissue lipid mobilised at each developmental stage and Table 3.66 gives the analysis of variance of these data. Both the treatments and the day of development had a significant effect on the variance of the lipid released. The general trend of the percentage of lipid mobilised at each stage of embryonic development was similar for the three treatments (Figure 3.23). The lipid release for all three treatments was highest at days 14 and 22 of development with a decrease at days 16 or 18 (see Figure 3.23 for statistical differences) in accordance with experiment B. Both doses of glucagon significantly increased the percentages of lipid mobilised at days 14 and 22.

However, the tissue was insensitive to both glucagon concentration at Day 16. Only the highest concentration of glucagon (1 μg/ml) induced an increase in lipid mobilisation compared to the controls' on day 18 which suggests that the sensitivity of adipose tissue to glucagon increases from day 16 to the hatching period. Tables 3.67, 3.68, 3.69 and 3.70 show the fatty acid profiles of the incubation medium FFA for the three different treatments and also of the adipose tissue TG before incubation. The fatty acid composition of the tissue TG did not change significantly during the 1h incubation in all cases (data not shown). As in experiments A and B, the proportions of long chain PUFA were higher in the medium FFA than in the adipose tissue TG. Medium/tissue ratios were calculated for each fatty acid (as described in *3.3.2*) and compared by an analysis of variance. The day of development was a source of variation for the ratios of all the major fatty acids except 16:1, 18:0 and 18:3. This analysis also showed that the treatments were a significant source of variation only for the medium/tissue ratios of the very long chain PUFA (20:4, 20:5 and 22:6). The day-treatment interactions were not significant for any ratio except the one for 20:5 (Table 3.73). The presence of glucagon was associated with significant reductions in the medium/tissue ratios of the long-chain PUFA (20:4, 20:5, 22:6) compared to the control. However, even in the presence of glucagon, the preferential release of 20:4, 20:5, 22:5 and 22:6 from the tissue TG into the medium, although attenuated was still consistently observed. The treatments had no effect on the ratios of other fatty acids on day 14, day 16 and day 18 (Tables 3.71 and 3.72), although both doses of glucagon significantly decreased the proportion of 18:0 in the medium on day 22. Figure 3.24 shows the log. of the ratios for the control incubations at each day of development studied. As in Figure 3.22, the preferential release of long chain PUFA (18:3, 20:4, 20:5, 22:5 and 22:6) was observed using tissue obtained throughout embryonic development.

3.3.4. Composition of fatty acids mobilised in vitro for adipose tissue obtained from embryos of the wild pheasant (Phasianus colchicus)

A similar study to that described for experiment A (section *3.3.1*) was designed using adipose tissue obtained from pheasant embryos. The limited number of eggs available only allowed an investigation at one developmental stage. The eggs were opened at day 16 of development, the total embryonic period being 24-26 days. Table 3.74 gives general characteristics of the adipose tissues collected (method as in *2.1.2*). Adipose tissue from pheasant embryos consisted mainly of triacylglycerol (97.4% of total lipids). The adipose tissues were incubated for 1 hour. The results (Table 3.75) shows again that the long chain PUFA were preferentially released in the incubation medium with proportions of DHA and AA higher in the medium FFA than in the adipose tissue TG (1.7 and 2.7-fold respectively, Figure 3.25).

3.3.5.Summary

The conclusions of this section, in which the mobilisation of fatty acids from adipose tissue TG has been studied during the *in vitro* incubation of adipose tissue pieces, are summarised below.

(i) The basal rate (i.e. in the absence of lipolytic agonists) of TG mobilisation measured by the release of FFA into the incubation medium and expressed as the percentage of tissue TG-fatty acid released, was relatively high using tissue at an earlier stage of adipose growth (i.e. day 14) but was lower at days 16 and 18, increasing again after hatching.

(ii) The tissue was sensitive to glucagon stimulation of TG mobilisation at day 14 but this sensitivity appeared to be lost by day 16 and then re-acquired by days 18 and 22.

(iii) A major finding was that C20 and C22 polyunsaturates (i.e. 20:4, 20:5, 22:5 and 22:6) were *preferentially mobilised from the tissue*. As a result, the proportion of these fatty acids measured in the medium FFA was consistently greater than the original proportion in the tissue TG; typically 2-3-fold greater in the case of DHA, although in some incubations there was a 5-7-fold preferential release of DHA.

This preferential release was found at all developmental stages, during both 1 and 24h incubations, in the presence and absence of glucagon, and in adipose tissue for both chick and pheasant embryos.

(iv) Although this prefenrential release was still apparent when glucagon was present, there was a general tendency, not always statistically significant, for the extent of preferential release to be somewhat reduced in the presence of this hormone.

Thus these *in vitro* studies are consistent with the results *in vivo* (section *3.1.5*) which suggest that DHA may be preferentially moilised from the adipose tissue of the chick embryo. The fact that such a process, occuring *in vivo* would result in the preferential release of DHA into the bloodstream as the free fatty acid, a form in which the fatty acid may be taken up by the embryonic brain, is a topic considered at some length in the Discussion section.

Table 3.61: Fatty acid compositions (% of total fatty acid) of adipose tissue triacylglycerol (AT-TG) and free fatty acid of incubation medium at day 16 of development (experiment A). Means ± S.E., n=4. Statistical comparison between tissue and incubation medium: $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

Fatty acids	% in AT-TG before incubation	% in medium FFA
16:0	28.5 ± 0.35	27.21 ± 0.98
16:1	3.26 ± 0.14	6.25 ± 0.66^{b}
18:0	8.15 ± 0.57	9.05 ± 0.46
18:1	39.97 ± 1.55	23.38 ± 0.95^{c}
18:2	14.09 ± 1.05	11.82 ± 0.99
18:3	0.68 ± 0.05	0.95 ± 0.05^{b}
20:4	2.19 ± 0.11	5.59 ± 0.52^{c}
20:5	0.10 ± 0.00	0.85 ± 0.08^{c}
22:5	0.18 ± 0.02	0.31 ± 0.11
22:6	2.43 ± 0.22	12.88 ± 0.71^{c}

Figure 3.21: The amount of fatty acid released into the medium from incubated adipose tissues at various stages of embryonic development. The percentage release was calculated as : Amount of fatty acid in the medium divided by the sum of the amounts of fatty acid in the medium and in adipose TG after incubation. Values are means ± S.E. of 4 replicates. Statistical differences between 1h-24h incubation period: at $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$. Statistical differences between 1h incubation day 18 vs day 14, and 1 hour day 22 vs day 14; 24 hours day 18 vs day 14, 24 hours day 22 vs day 14: values differing at $^{*}P<0.05$, $^{**}P<0.01$.

1h incubation
24h incubation
% lipid mobilised
3
2.5
2
1.5
1
0.5
0
c
*
**
c
*
**
b
D14
D18
D22
Days of development

Table 3.62: Fatty acid compositions (% of total fatty acid) of adipose tissue TG and incubation medium FFA at day 14 of development from experiment B. Means ± S.E., n=4. Statistical differences between tissue and incubation medium: at $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$; Statistical differences between incubation medium at 1h versus 24h: at $^{*}P<0.05$.

Fatty acids	% in AT-TG before incubation	% in medium FFA, 1 hour incubation	Ratio %FA (1h) medium / AT	% in medium FFA, 24 hour incubation	Ratio %FA (24h) medium / AT
16:0	29.20 ± 0.14	19.52 ± 0.23^{c}	0.67	19.76 ± 0.13^{c}	0.68
16:1	3.57 ± 0.09	3.15 ± 0.12	0.88	3.26 ± 0.13	0.90
18:0	7.37 ± 0.11	12.04 ± 0.39^{c}	1.63	10.75 ± 0.28^{c*}	1.46
18:1	34.99 ± 0.26	30.13 ± 0.57^{c}	0.86	32.12 ± 0.45^{b*}	0.92
18:2	16.94 ± 0.18	16.20 ± 0.50	0.96	17.47 ± 0.70	1.08
18:3	1.31 ± 0.02	3.46 ± 0.05^{c}	2.64	3.24 ± 0.07^{c*}	2.47
20:4	2.33 ± 0.07	4.41 ± 0.27^{c}	1.89	3.81 ± 0.08^{c}	1.64
20:5	0.16 ± 0.01	0.83 ± 0.04^{c}	5.19	0.74 ± 0.11^{b}	4.63
22:5	0.64 ± 0.03	1.11 ± 0.01^{c}	1.73	0.98 ± 0.07^{b}	1.53
22:6	2.87 ± 0.12	8.23 ± 1.01^{b}	2.87	7.08 ± 0.48^{c}	2.47

Table 3.63: Fatty acid compositions (% of total fatty acid) of adipose tissue TG and incubation medium FFA at Day 16 of development from experiment B. Means ± S.E., n=4. Statistical differences between tissue and incubation medium: at $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

Fatty acids	% in AT-TG before incubation	% in medium FFA, 1 hour incubation	Ratio %FA (1h) medium / AT
16:0	29.04 ± 0.36	15.04 ± 0.86^{c}	0.52
16:1	3.48 ± 0.15	2.22 ± 0.12^{c}	0.64
18:0	7.69 ± 0.10	13.51 ± 0.51^{c}	1.76
18:1	38.29 ± 1.10	32.25 ± 0.49^{b}	0.84
18:2	14.28 ± 0.56	16.92 ± 0.43^{a}	1.18
18:3	1.05 ± 0.04	3.56 ± 0.21^{c}	3.39
20:4	2.15 ± 0.03	4.70 ± 0.30^{c}	2.19
20:5	0.13 ± 0.01	0.97 ± 0.05^{c}	7.46
22:5	0.39 ± 0.02	1.40 ± 0.04^{c}	3.59
22:6	2.79 ± 0.02	8.73 ± 0.68^{c}	3.13

Table 3.64: Fatty acid compositions (% of total fatty acid) of adipose tissue TG and incubation medium FFA at day 18 of development from Experiment B. Means ± S.E., n=4. Statistical differences between tissue and incubation medium: at $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$; Statistical differences between tissue at 1h versus 24h: at $^{x}P<0.05$, $^{y}P<0.01$; Statistical differences between incubation medium at 1h versus 24h: at $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Fatty acids	% in AT-TG before incubation	% in AT-TG after 24 hour incubation	% in medium FFA, 1 hour incubation	Ratio %FA (1h) medium / AT	% in medium FFA, 24 hour incubation	Ratio %FA (24h) medium / AT
16:0	27.49 ± 0.29	28.87 ± 0.19	21.03 ± 1.49^{b}	0.77	21.85 ± 0.86^{c}	0.79
16:1	4.29 ± 0.08	3.88 ± 0.14^{x}	3.14 ± 0.49	0.73	3.44 ± 0.05^{c}	0.80
18:0	7.34 ± 0.10	7.38 ± 0.21	13.13 ± 0.92^{c}	1.79	9.90 ± 0.22^{c*}	1.35
18:1	39.22 ± 1.60	40.15 ± 0.69	28.91 ± 1.66^{b}	0.74	34.20 ± 0.59^{a*}	0.87
18:2	15.84 ± 1.04	15.03 ± 0.50	15.82 ± 0.54	1.00	16.65 ± 0.48	1.07
18:3	1.43 ± 0.12	1.18 ± 0.06	3.32 ± 0.19^{c}	2.32	2.94 ± 0.06^{c}	2.06
20:4	1.60 ± 0.07	1.31 ± 0.05^{x}	4.44 ± 0.29^{c}	2.78	3.39 ± 0.09^{c**}	2.12
20:5	0.10 ± 0.01	0.08 ± 0.004	0.83 ± 0.07^{c}	8.3	0.66 ± 0.02^{c}	6.6
22:5	0.40 ± 0.06	0.29 ± 0.03	1.40 ± 0.02^{c}	3.5	0.92 ± 0.04^{c***}	2.3
22:6	1.69 ± 0.09	1.13 ± 0.07^{y}	6.78 ± 0.46^{c}	4.01	5.34 ± 0.15^{c*}	3.16

Table 3.65: Fatty acid compositions (% of total fatty acid) of adipose tissue TG and incubation medium FFA at day 22 of development from experiment B. Means ± S.E., n=4. Statistical differences between tissue and incubation medium: at $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$; Statistical differences between incubation medium at 1h versus 24h: at $^{*}P<0.05$, $^{***}P<0.001$.

Fatty acids	% in AT-TG before incubation	% in medium FFA, 1 hour incubation	Ratio %FA (1h) medium / AT	% in medium FFA, 24 hour incubation	Ratio %FA (24h) medium / AT
16:0	28.92 ± 0.14	21.15 ± 1.53^{b}	0.73	16.85 ± 0.52^{c*}	0.59
16:1	3.66 ± 0.11	3.88 ± 0.21	1.06	3.13 ± 0.28	0.86
18:0	7.40 ± 0.15	8.61 ± 0.91	1.16	11.14 ± 0.43^{c*}	1.51
18:1	39.19 ± 1.26	39.11 ± 3.20	1.00	37.99 ± 0.7	0.97
18:2	17.32 ± 1.02	17.73 ± 0.89	1.02	20.76 ± 0.48^{a*}	1.20
18:3	1.41 ± 0.11	2.86 ± 0.13^{c}	2.03	3.69 ± 0.04^{c***}	2.62
20:4	0.92 ± 0.07	2.62 ± 0.51^{a}	2.85	2.74 ± 0.24^{c}	2.98
20:5	0.04 ± 0.006	0.48 ± 0.07^{c}	12.00	0.57 ± 0.07^{c}	14.25
22:5	0.19 ± 0.04	0.58 ± 0.12^{a}	3.05	0.87 ± 0.06^{c}	4.58
22:6	0.34 ± 0.01	2.53 ± 0.88^{a}	7.44	1.69 ± 0.27^{b}	4.97

Figure 3.22: Log_{10} of ratios (% FA in medium FFA / % FA in adipose tissue TG) at various stages of embryonic development. Values for experiment B, 1 hour incubation.

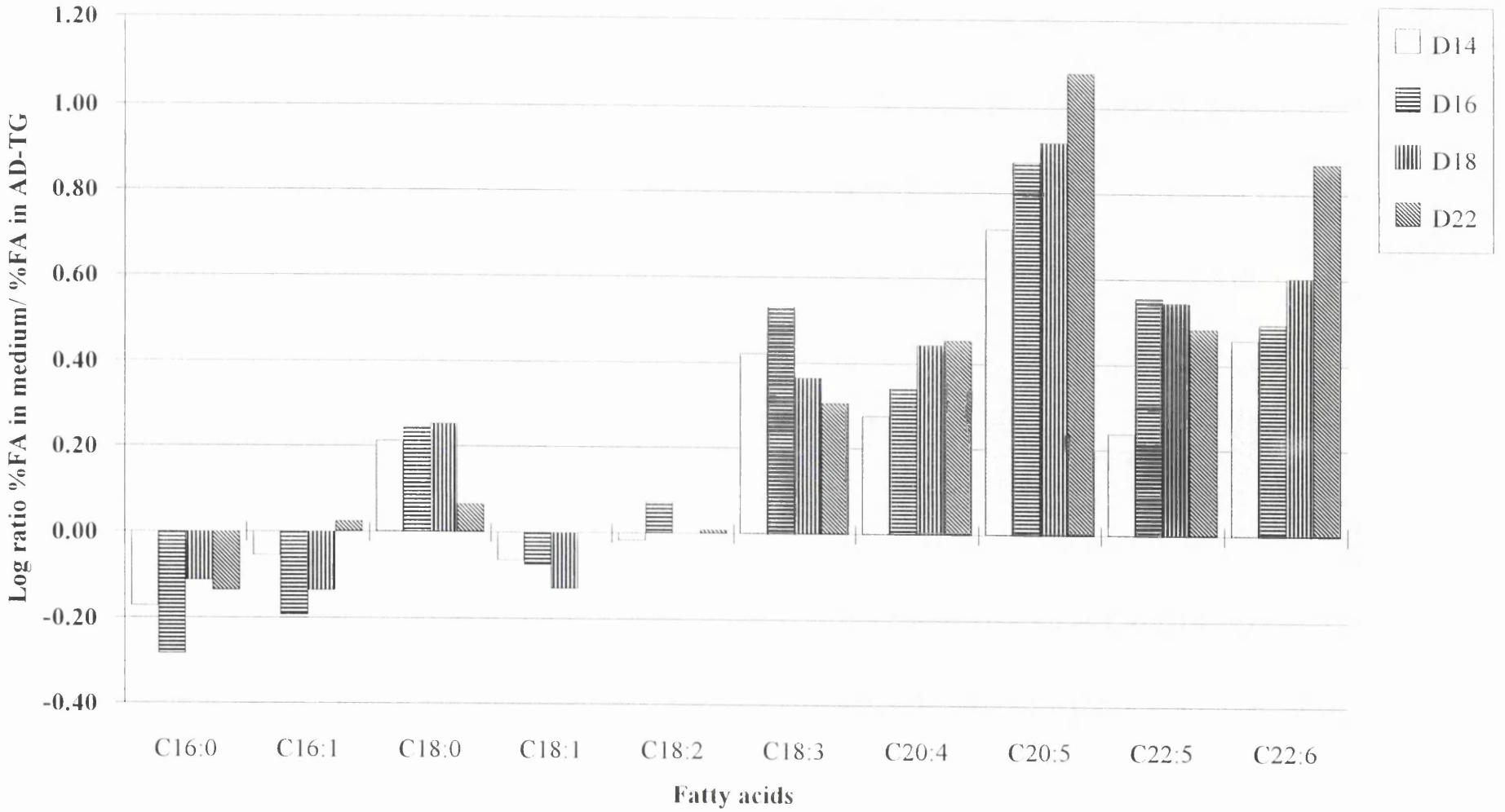

1.20
1.00
0.80
0.60
0.40
0.20
0.00
-0.20
-0.40
Log ratio %FA in medium/ %FA in AD-TG
D14
D16
D18
D22
C16:0
C16:1
C18:0
C18:1
C18:2
C18:3
C20:4
C20:5
C22:5
C22:6
Fatty acids

Figure 3.23: Percentage of tissue TG mobilised to form medium FFA from incubated adipose tissue pieces obtained at various stages of embryonic development from experiment C. Percentage calculated as : Amount of FFA in medium divided by the sum of the amounts of FFA in medium and in adipose TG after incubation. Values are mean of 4 replicates ± S.E.M.. Statistical differences between treatment and control at each time point: ^{a}P<0.05, ^{b}P<0.02, ^{c}P<0.01. Abbreviation: gluc refers to glucagon. Statistical comparison treatment at a time point and D14s' value for the same treatment: *P<0.05.

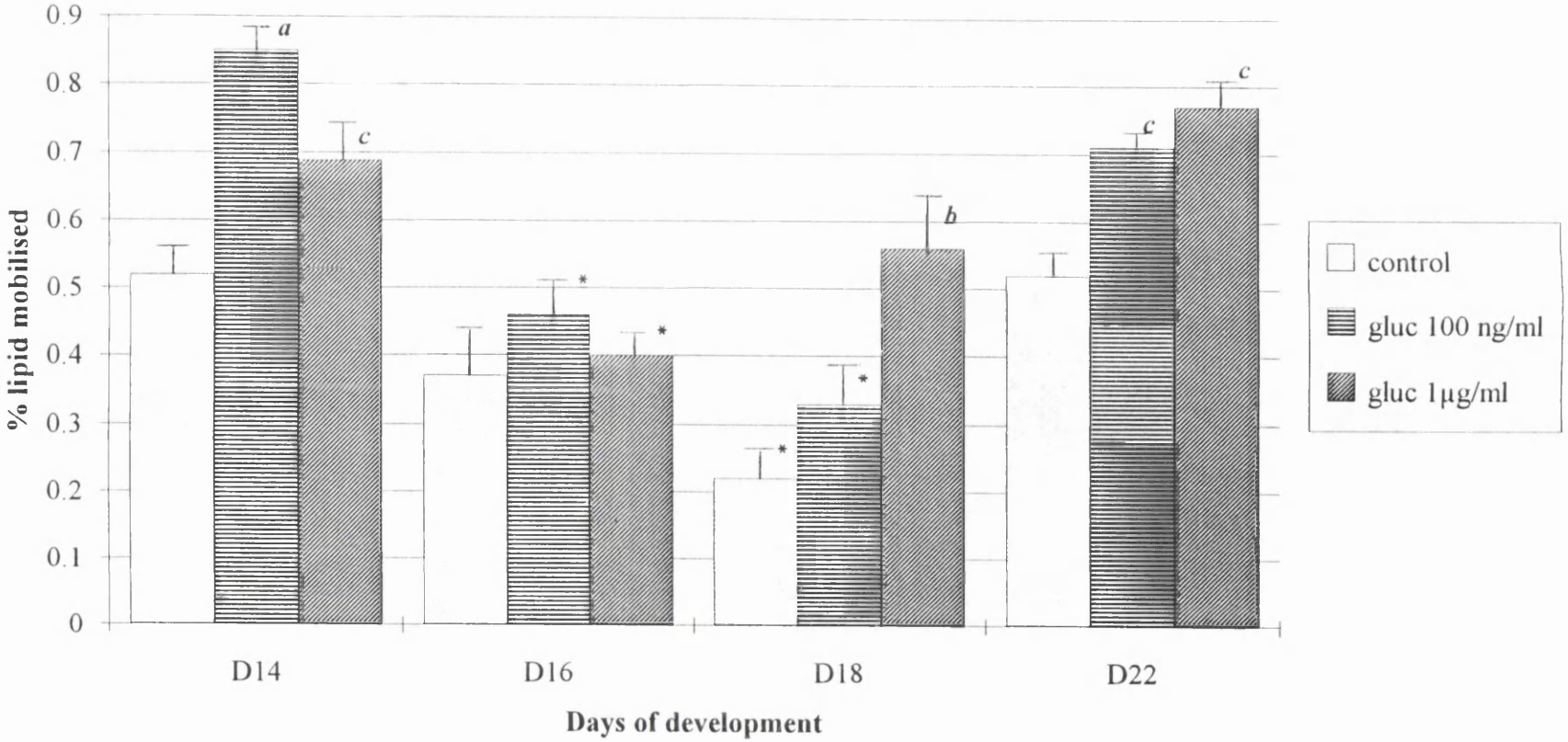

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
% lipid mobilised
a
c
*
*
*
b
c
c
D14
D16
D18
D22
Days of development
control
gluc 100 ng/ml
gluc 1µg/ml

Table 3.66: Analysis of variance of % tissue lipid mobilised (Amount of FFA in medium divided by the sum of amounts of FFA in medium and in adipose tissue TG after incubation), DF : degree of freedom.

	% lipid released		
Source of variation	DF	F	P
Day	3	15.34	P<0.001
Treatment	2	8.86	P<0.01
Day*treatment	6	1.715	NS

Table 3.67: Fatty acid compositions (% of total fatty acid) of adipose tissue TG and incubation medium FFA (1 hour) at day 14 of development from experiment C. Means ± S.E., n=4. Statistical comparison between tissue and incubation medium: $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

Fatty acids	% in AT-TG before incubation	% in medium, control	% in medium, glucagon 100 ng/ml	% in medium, glucagon 1 µg/ml
16:0	28.27 ± 0.51	20.52 ± 0.80^{c}	21.04 ± 0.50^{c}	21.85 ± 0.35^{c}
16:1	3.18 ± 0.10	3.68 ± 0.19	3.29 ± 0.09	3.45 ± 0.23
18:0	7.69 ± 0.23	11.19 ± 1.59	13.42 ± 1.21^{b}	13.00 ± 0.73^{c}
18:1	37.41 ± 0.77	31.82 ± 1.55^{a}	30.09 ± 1.10^{b}	29.79 ± 1.08^{b}
18:2	14.42 ± 0.37	17.77 ± 0.94^{a}	16.74 ± 0.24^{b}	16.92 ± 0.27^{b}
18:3	0.89 ± 0.28	3.73 ± 0.33^{c}	4.50 ± 0.25^{c}	4.48 ± 0.30^{c}
20:4	1.90 ± 0.19	2.59 ± 0.15^{a}	2.50 ± 0.09^{a}	2.43 ± 0.15
20:5	0.20 ± 0.02	0.95 ± 0.10^{c}	1.01 ± 0.06^{c}	0.95 ± 0.06^{c}
22:5	0.49 ± 0.07	1.05 ± 0.09^{c}	0.98 ± 0.06^{c}	0.96 ± 0.06^{c}
22:6	4.79 ± 0.53	5.70 ± 0.20^{a}	5.51 ± 0.64	5.20 ± 0.38

Table 3.68: Fatty acid compositions (% of total fatty acid) of adipose tissue TG and incubation medium FFA (1 hour) at day 16 of development from experiment C. Means ± S.E., n=4. Statistical comparison between tissue and incubation medium: $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

Fatty acids	% in AT-TG before incubation	% in medium, control	% in medium, glucagon 100 ng/ml	% in medium, glucagon 1 µg/ml
16:0	28.96 ± 0.34	20.90 ± 0.26^{c}	21.83 ± 0.24^{c}	22.16 ± 0.23^{c}
16:1	3.28 ± 0.23	3.89 ± 0.27	3.87 ± 0.20	3.88 ± 0.30
18:0	8.85 ± 0.51	12.99 ± 1.25^{a}	11.36 ± 0.99	11.33 ± 1.38
18:1	38.94 ± 1.26	30.07 ± 1.72^{b}	32.09 ± 1.30^{b}	29.57 ± 1.23^{b}
18:2	13.39 ± 1.08	16.99 ± 0.54^{a}	17.62 ± 0.40^{a}	18.01 ± 0.64^{b}
18:3	1.15 ± 0.13	3.78 ± 0.19^{c}	3.95 ± 0.26^{c}	4.00 ± 0.38^{c}
20:4	1.50 ± 0.02	2.35 ± 0.19^{c}	2.11 ± 0.11^{c}	2.18 ± 0.13^{c}
20:5	0.14 ± 0.00	0.79 ± 0.08^{c}	0.80 ± 0.09^{c}	0.84 ± 0.09^{c}
22:5	0.38 ± 0.02	0.97 ± 0.04^{c}	0.83 ± 0.09^{b}	0.89 ± 0.06^{c}
22:6	2.71 ± 0.12	6.15 ± 0.83^{b}	4.49 ± 0.41^{b}	6.07 ± 0.82^{b}

Table 3.69: Fatty acid compositions (% of total fatty acid) of adipose tissue TG and incubation medium FFA (1 hour) at day 18 of development from experiment C. Means ± S.E., n=4. Statistical comparison between tissue and incubation medium: $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

Fatty acids	% in AT-TG before incubation	% in medium, control	% in medium, glucagon 100 ng/ml	% in medium, glucagon 1 µg/ml
16:0	29.07 ± 0.25	23.36 ± 0.26^{c}	23.66 ± 1.36^{b}	23.30 ± 0.72^{c}
16:1	3.92 ± 0.09	4.22 ± 0.38	3.78 ± 0.36	3.74 ± 0.14
18:0	8.01 ± 0.22	14.56 ± 2.41^{a}	14.05 ± 1.47^{b}	13.0 ± 1.33^{a}
18:1	40.52 ± 0.55	29.37 ± 2.09^{c}	31.06 ± 0.74^{c}	30.00 ± 0.32^{c}
18:2	14.21 ± 0.17	15.61 ± 0.21^{b}	16.37 ± 0.44^{b}	17.00 ± 0.16^{c}
18:3	1.25 ± 0.04	4.02 ± 0.39^{c}	4.03 ± 0.8^{c}	4.25 ± 0.26^{c}
20:4	1.07 ± 0.14	2.20 ± 0.11^{c}	1.94 ± 0.01^{c}	2.16 ± 0.12^{b}
20:5	0.08 ± 0.01	0.83 ± 0.11^{c}	0.78 ± 0.04^{c}	0.89 ± 0.045^{c}
22:5	0.21 ± 0.02	0.95 ± 0.09^{c}	0.78 ± 0.07^{c}	0.96 ± 0.03^{c}
22:6	0.94 ± 0.17	3.67 ± 0.40^{c}	2.43 ± 0.05^{c}	3.70 ± 0.52^{c}

Table 3.70: Fatty acid compositions (% of total fatty acid) of adipose tissue TG and incubation medium FFA (1 hour) at day 22 of development from experiment C. Means ± S.E., n=4. Statistical comparison between tissue and incubation medium: $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

Fatty acids	% in AT-TG before incubation	% in medium, control	% in medium, glucagon 100 ng/ml	% in medium, glucagon 1 µg/ml
16:0	28.95 ± 1.17	17.91 ± 0.74^{c}	18.86 ± 0.49^{c}	17.89 ± 0.08^{c}
16:1	2.51 ± 0.84	3.03 ± 0.20	3.64 ± 0.14	3.33 ± 0.20
18:0	6.84 ± 0.57	15.04 ± 0.58^{c}	10.35 ± 0.51^{b}	11.41 ± 0.43^{c}
18:1	38.93 ± 1.08	34.70 ± 1.02^{a}	38.64 ± 0.62	36.44 ± 0.56
18:2	19.38 ± 1.9	18.45 ± 0.58	21.19 ± 0.84	21.23 ± 1.08
18:3	1.13 ± 0.38	3.70 ± 1.17	1.90 ± 1.01	4.28 ± 0.02^{c}
20:4	0.76 ± 0.07	2.36 ± 0.27^{c}	2.04 ± 0.04^{c}	1.98 ± 0.07^{c}
20:5	0.04 ± 0.01	0.92 ± 0.04^{c}	0.69 ± 0.04^{c}	0.76 ± 0.01^{c}
22:5	0.17 ± 0.04	0.94 ± 0.06	0.67 ± 0.10	0.70 ± 0.01
22:6	0.62 ± 0.21	2.14 ± 0.43^{a}	1.49 ± 0.14^{a}	1.33 ± 0.29

Table 3.71: Ratio (% fatty acid in incubation medium FFA / % fatty acid in adipose tissue TG) after incubation (1h) for tissue obtained at days 14 and 16 of development. Means ± SE, n=4. Statistical difference between treatment and control within the same day : $^{a}P<0.05$.

	16:0	16:1	18:0	18:1	18:2	18:3	20:4	20:5	22:5	22:6
day 14										
Control	0.74 ± 0.02	1.08 ± 0.11	1.52 ± 0.23	0.84 ± 0.05	1.18 ± 0.03	3.16 ± 0.29	1.43 ± 0.14	4.98 ± 0.91	1.85 ± 0.15	1.54 ± 0.15
Glucagon 100 ng/ml	0.76 ± 0.03	1.04 ± 0.04	1.73 ± 0.15	0.82 ± 0.03	1.15 ± 0.03	3.44 ± 0.19	1.14 ± 0.06	3.66 ± 0.39	1.68 ± 0.23	1.15 ± 0.12
Glucagon 1 µg/ml	0.78 ± 0.01	1.09 ± 0.02	1.79 ± 0.11	0.83 ± 0.03	1.09 ± 0.04	3.73 ± 0.25	1.18 ± 0.07	3.94 ± .37	1.46 ± 0.13	1.14 ± 0.12
day 16										
Control	0.75 ± 0.02	1.09 ± 0.06	1.66 ± 0.17	0.78 ± 0.05	1.10 ± 0.06	3.04 ± 0.26	1.69 ± 0.18	6.00 ± 0.83	2.45 ± 0.29	2.28 ± 0.30
Glucagon 100 ng/ml	0.79 ± 0.01	1.17 ± 0.07	1.42 ± 0.14	0.83 ± 0.03	1.15 ± 0.04	2.90 ± 0.18	1.36 ± 0.06	5.66 ± 0.46	2.10 ± 0.14	1.59^{a} ± 0.07
Glucagon 1 µg/ml	0.76 ± 0.01	1.08 ± 0.06	1.63 ± 0.20	0.82 ± 0.3	1.06 ± 0.03	2.97 ± 0.16	1.51 ± 0.06	5.75 ± 0.80	2.13 ± 0.20	1.88^{a} ± 0.11

Table 3.72: Ratio (% fatty acid in incubation medium FFA / % fatty acid in adipose tissue TG) after incubation (1h) for tissue obtained at days 18 and 22 of development. Means ± SE, n=4. Statistical difference treatment and control within the same day : $^{a}P<0.05$.

	16:0	16:1	18:0	18:1	18:2	18:3	20:4	20:5	22:5	22:6
D18										
Control	0.81 ± 0.00	1.11 ± 0.05	1.85 ± 0.28	0.72 ± 0.05	1.13 ± 0.02	3.24 ± 0.52	2.20 ± 0.16	8.58 ± 0.85	3.29 ± 0.25	2.93 ± 0.49
Glucagon 100 ng/ml	0.84 ± 0.05	1.02 ± 0.09	1.78 ± 0.17	0.79 ± 0.02	1.10 ± 0.03	3.08 ± 0.16	1.47^{a} ± 0.11	5.74^{a} ± 0.51	2.43^{a} ± 0.20	1.42^{a} ± 0.24
Glucagon 1 µg/ml	0.801 ± 0.03	0.96 ± 0.02	1.76 ± 0.14	0.79 ± 0.02	1.06 ± 0.06	2.94 ± 0.42	1.86 ± 0.21	7.55 ± 0.63	2.80 ± 0.07	2.39 ± 0.44
D22										
Control	0.64 ± 0.04	0.92 ± 0.08	1.85 ± 0.05	0.85 ± 0.02	1.21 ± 0.02	2.80 ± 0.88	2.50 ± 0.10	15.43 ± 0.52	4.30 ± 0.61	2.13 ± 0.37
Glucagon 100 ng/ml	0.65 ± 0.01	1.14^{a} ± 0.02	1.30^{a} ± 0.03	0.97^{a} ± 0.01	1.34^{a} ± 0.04	1.48 ± 0.79	2.40 ± 0.18	9.97^{a} ± 0.67	2.52^{a} ± 0.37	2.11 ± 0.20
Glucagon 1 µg/ml	0.63 ± 0.00	1.10 ± 0.03	1.47^{a} ± 0.05	0.92^{a} ± 0.01	1.27 ± 0.04	3.29 ± 0.13	2.23^{a} ± 0.04	11.7^{a} ± 1.1	2.94 ± 0.28	1.87 ± 0.07

Table 3.73: Analysis of variance of ratio (% FA in medium FFA / % FA in tissue TG), DF : degree of freedom.

Source of variation		**16:0**			**16:1**			**18:0**	
	DF	F	**P**	DF	F	**P**	DF	F	**P**
Day	3	30.44	**P<0.001**	3	1.10	**NS**	3	1.55	**NS**
Treatment	2	0.92	**NS**	2	0.63	**NS**	2	1.04	**NS**
Day*treatment	6	1.40	**NS**	6	1.99	**NS**	6	1.15	**NS**
		18:1			**18:2**			**18:3**	
	DF	F	**P**	DF	F	**P**	DF	F	**P**
Day	3	10.71	**P<0.001**	3	13.95	**P<0.001**	3	2.37	**NS**
Treatment	2	3.42	**P<0.05**	2	2.54	**NS**	2	1.45	**NS**
Day*treatment	6	0.92	**NS**	6	1.27	**NS**	6	1.32	**NS**
		20:4			**20:5**			**22:6**	
	DF	F	**P**	DF	F	**P**	DF	F	**P**
Day	3	44.48	**P<0.001**	3	74.26	**P<0.001**	3	7.53	**P<0.001**
Treatment	2	8.85	**P<0.01**	2	12.43	**P<0.001**	2	6.3	**P<0.05**
Day*treatment	6	1.34	**NS**	6	2.68	**P<0.05**	6	1.67	**NS**

Figure 3.24: Log_{10} ratio (% FA in medium FFA / % FA in initial adipose TG) for the control during various stages of embryonic development.

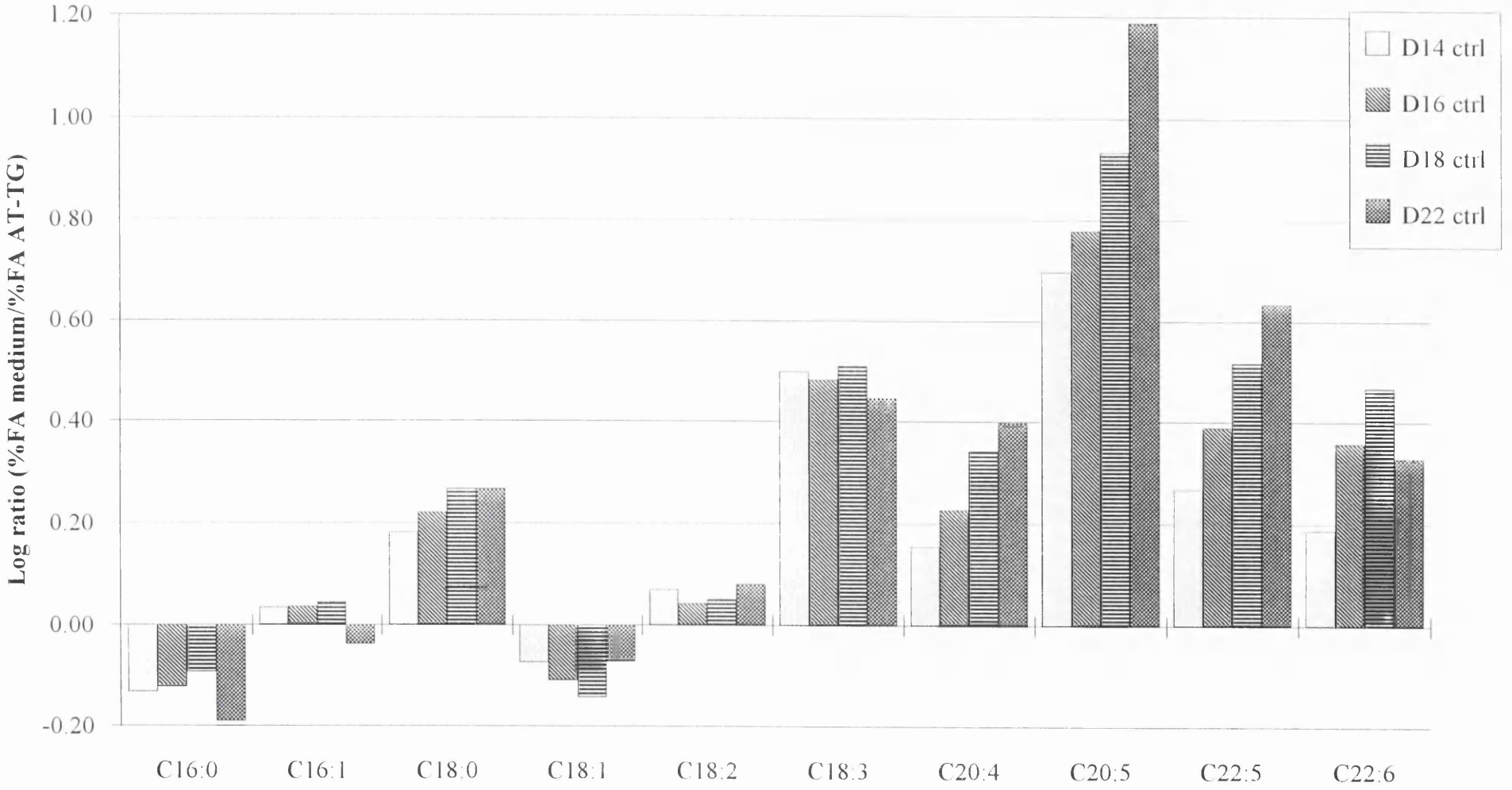

Log ratio (%FA medium/%FA AT-TG)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
-0.20
D14 ctrl
D16 ctrl
D18 ctrl
D22 ctrl
C16:0
C16:1
C18:0
C18:1
C18:2
C18:3
C20:4
C20:5
C22:5
C22:6

Table 3.74: Characteristics of pheasant (*Phasianus colchicus*) adipose tissue at day 16 of incubation. Values are means ± S.E., n=2.

Adipose depot wt (g)				Amount of lipid / depot (mg)				
	0.13 ± 0.01			29.5 ± 2.2				
	% of total lipids							
TG		97.4 ± 0.2						
PL		2.7 ± 0.2						
% FA	16:0	16:1	18:0	18:1	18:2	18:3	20:4	22:6
TG	29.1 ±0.0	5.0± 0.2	6.2± 0.0	39.3 ±1.0	15.1 ±1.2	1.2± 0.1	0.8± 0.0	2.7± 0.6
PL	28.7 ±0.9	ND	14.8 ±1.0	33.9 ±0.8	13.5 ±0.8	0.7± 0.1	4.5± 0.3	3.5± 0.3

Table 3.75: Fatty acid profiles of pheasant adipose TG and incubation medium FFA. Values are means of 2 separate replicates ± S.E. Statistical differences between tissue TG before incubation and incubation medium: $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

	% FA in AT-TG before incubation	% FA in medium after 1h incubation	Ratio %FA medium / %FA AT-TG
16:0	29.1 ± 0.03	21.28 ± 1.25^{c}	0.73
16:1	4.95 ± 0.17	4.34 ± 0.17^{a}	0.88
18:0	6.16 ± 0.2	13.19 ± 0.37^{c}	2.14
18:1	39.26 ± 1.02	31.04 ± 0.89^{c}	0.79
18:2	15.05 ± 1.19	16.01 ± 0.75	1.06
18:3	1.16 ± 0.06	4.36 ± 0.22^{c}	3.76
20:4	0.81 ± 0.05	2.22 ± 0.12^{c}	2.74
20:5	0.15 ± 0.02	1.43 ± 0.09^{c}	9.53
22:6	2.74 ± 0.60	4.76 ± 0.5^{a}	1.74

Figure 3.25: Log (ratio %FA in medium FFA/%FA in adipose tissue TG) for pheasant adipose tissue, 1h incubation.

Log ratio (%FA medium FFA/% FA in tissue TG)
1
0.8
0.6
0.4
0.2
0
-0.2
C16:0
C16:1
C18:0
C18:1
C18:2
C18:3
C20:4
C20:5
C22:6

DISCUSSION

4.1-RECENT VIEWS REGARDING THE IMPORTANCE OF PUFA IN NEURAL FUNCTION

The importance of DHA for the functional development of both the brain and the retina was described in section *1.4*. During the past year an increasing number of studies have focused on the importance of *n-3* fatty acids for human health in new areas such as mental health and illness. Adams *et al* (1995) showed a significant correlation between the erythrocyte PL ratio of AA to EPA and the severity of mental depression. This ratio was high in depressed patients and could not be explained by different dietary intakes of EPA. Laugharne *et al* (1995) and Glen *et al* (1994) reported a depletion of both AA and DHA in red cell membrane of schizophrenic patients. In these studies no evidence was given to indicate if *n-3* fatty acid depletion was a symptom or a cause of the illness. Salem *et al* (1995) reported the effect of alcoholism on blood and tissue EFA levels. They reported that alcoholics had losses in blood PUFA due to alcohol induced EFA catabolism. Results obtained from various mammals after chronic alcohol exposure showed a decrease of DHA level in neural tissues which correlated with the functional impairments of these tissues. The level of 22:5*n-6* increased in the brain to compensate for the loss of DHA. In the three cases cited above, decreases in the severity of symptoms could be obtained by nutritional therapy (i.e. by increasing the dietary EFA levels).

Makrides *et al* (1995) have provided more evidence with regards to DHA supplementation of infant milk formula. They reported the effect on the visual acuity of supplemented infants at 16 and 30 weeks of age as an indicator of neural maturation. They showed that this supplementation resulted in an improvement in visual acuity, compared to babies with standard formula, to match that of fully breastfed infants. However, the long term outcomes still have to be studied. Connor *et al* (1995) looked at the problem at a different angle as they supplemented the diet of women from their 26th to 35th week of pregnancy. This supplementation significantly increased the erythrocyte DHA level of both the mother and the new-born infant. At a recent major conference on the role of fatty acids in human health (ISSFAL, Maryland, USA, June 1995), the views of four leading researchers in this area regarding the desirability to ensure, by supplementation if necessary, that the developing human brain is optimally supplied with DHA, were discussed in a workshop session. Hornstra *et al* (1995) reported that maternal stores of DHA were dramatically depleted after each pregnancy, emphasising that the first child of a given woman has generally a better DHA status than that of her following children. This study indicated that the maternal DHA dietary intake was not sufficient to guarantee an adequate DHA supply to the fetus. Furthermore Morley (1995) compared the developmental performances of premature infants fed maternal milk to that fed milk formula. She provided evidence that breast-

fed children performed significantly better at both 18 months post-term and at 7.5 to 8 years of age. Crawford (1995) advised that diets of women should be enriched in *n-6* and *n-3* long chain PUFA before and during pregnancy, that the milk formula in current use for preterm infants is inadequate, with too much emphasis on 18:2*n-6,* and finally that bottle-fed infants had an 8 point IQ deficit at 8 years of age and a significantly reduced cortical DHA level compared to that of breast fed children. These studies therefore encouraged breast feeding and the enrichment of milk formula in *n-6* and *n-3* long chain PUFA. However, Innis (1995) reported several studies where no differences in visual acuity or developmental scores were found between term gestation breast fed and bottle fed babies. The milk formula used in these studies did not contain AA or DHA. This report indicated that information on the potential dietary essentiality of AA and DHA for very premature infants is limited and the outcomes are still unclear. DHA inclusion in formulas reduced scores on some developmental tests in both premature and term gestation infants. Premature infant growth was also slowed. This report concluded that AA and DHA supplementations into infant formula should be more carefully studied with the knowledge that these fatty acids are potent metabolites which may have untoward effects on infant growth and development when fed from sources other than human milk. Thus complete consensus in this area remains to be achieved.

In all cases cited above, the role of nutritional therapies was emphasised. In the case of the human infant, at least two strategies of n-3 supplementation are available: supplementation of the baby's diet during its post-natal life or supplementation of the mother's diet during pregnancy. To be able to provide DHA both in a molecular form (i.e. TG vs. PL, position of DHA on glycerol backbone, nature of other fatty acids present) and at a time during development (e.g. early pregnancy, late pregnancy, lactation) which favours the delivery of this fatty acid to the developing neural tissues, we need to understand the route and mechanism by which DHA is delivered to these tissues. This project was aimed at improving the knowledge of DHA delivery to the brain of the developing embryo. The chick embryo appeared to be a suitable model to study this subject as all embryonic development occurs in a closed system using lipid pre-packaged in the yolk as the sole source of fatty acids. At a later stage in this discussion (section *4.2* and Figure 4.1) a hypothesis is presented, based on the results described in this thesis, suggesting a unique route by which DHA is transferred from the yolk to the brain. Speculations regarding the possible relevance of concepts obtained using the chick embryo system to the mammalian and human situations are discussed in section *4.5*.

4.2-TRANSFER OF FATTY ACIDS FROM THE YOLK COMPLEX TO THE MAJOR ORGANS OF THE CHICK EMBRYO

4.2.1. General aim of this section of the study

The rationale behind the experiments described in section *3.1.* was, by measuring the amounts and proportions of the major fatty acyl components of the lipids of the yolk, the whole embryo, the major tissues and the blood plasma throughout embryonic development, to provide a detailed picture of the rate of transfer of fatty acyl moieties from the yolk and the partitioning of these fatty acids between the embryonic tissues. A key aim was to delineate the route and mechanism by which DHA is transported from the yolk to the developing brain.

4.2.2. Transfer from the yolk sac membrane to the embryonic circulation

A major advantage in using the avian embryo to study lipid transfer stems from the fact that lipids and their constituent fatty acids which are required by the developing embryo are supplied "pre-packaged" in the yolk. Moreover, the avian embryo system has a very low capacity for the *de novo* synthesis of fatty acids from carbohydrate. Thus the fatty acids which accumulate in the embryo lipids are almost entirely derived from the yolk; the rate of this incorporation will therefore depend on the kinetics of transfer from the yolk and the rate of β-oxidation in the tissues. An additional factor regulating the levels of particular fatty acids in the lipids of the embryonic tissues will be the action of desaturation/elongation systems. For example, high levels of Δ6-desaturase are expressed in the yolk sac membrane and tissues of the chick embryo and the major function of this enzyme would appear to be in the conversion of 18:2 to AA (Noble and Cocchi, 1990). Although the synthesis of DHA via Δ6-desaturase has been demonstrated in the chick embryo (Miyamoto *et al*, 1967), this process may be of limited quantitative importance since, as indicated in Table 3.1, the amounts of available 18:3 substrate in the yolk is very low. The data in Table 3.1 also indicate that the amounts of 18:2 in the yolk are 13-16 times higher than the amounts of 18:3, thus potentially providing a very effective substrate competition for the Δ6-desaturase enzyme. Experimental evidence for the view that desaturation/elongation of 18:3 does *not* make a significant contribution to the DHA content of the embryo has been provided by the studies of Anderson *et al* (1990) and Lin *et al* (1991). Thus it would appear that, under normal conditions, the DHA present in the yolk lipids is by far the major source of DHA for the embryo; a fact which greatly facilitates the interpretation of the data presented in section *3.1.*

With regard to the transfer of total lipid from the yolk during embryonic development (Figure 3.1), the amount of lipid transferred from the yolk complex prior to day 12 of development was very low. However, the period around day 12 marks a phase of major metabolic transition, characterised, as illustrated in Figure 3.1, by a dramatic increase in the rate of lipid transfer, in agreement with previous work (Noble and Cocchi, 1990). Thus, up to more than 80% of the initial yolk lipid content is transferred to the embryo between day 12 of embryogenesis and the first day of hatching (Figure 3.1). The proportions of the major lipid classes of the yolk complex, TG and PL expressed as % (w/w) of total lipid, remained approximately constant throughout development in line with the view that the bulk of the lipid is taken up from the yolk by the yolk sac membrane by phagocytosis in a relatively non-discriminatory fashion (Noble and Cocchi, 1990). However, in one of the experiments (experiment 1), the proportion of CE in the yolk complex increased dramatically between days 18 and 22 (Tables 3.4 and 3.5). Similarly, expression of the levels of the lipid classes in terms of the absolute amounts (mg/yolk complex) (Figure 3.2) showed that TG, PL and CHOL were removed from the yolk roughly in parallel, whereas the amount of CE in the yolk complex in one of the experiment (experiment 1) remained constant or increased. This may be explained firstly by the rapid esterification of CHOL to CE within the yolk sac membrane (Shand *et al*, 1993) and consequently by the recycling of CE from the liver, via the bile duct and the small intestine, back to the yolk (Noble and Cocchi, 1990). The differences between the two experiments may reflect different efficiencies in the recycling of CE.

Analysis of the fatty acid composition of the total lipid of the yolk complex during development (Tables 3.6 and 3.7) revealed, for the most part, a remarkable consistency in the relative proportions of the major fatty acids. A singular exception to this consistency was exhibited by DHA, whose proportion decreased significantly, by more than 50%, during the course of development. Furthermore, it is clear from Tables 3.8 and 3.9 that this decrease in the proportion of DHA occurs in PL, and not the TG, fraction. Presentation of the specific fatty acid content of the lipids of the yolk complex in terms of the percentage of the levels initially present in the yolk prior to incubation, allows the changes in the amounts of the different fatty acids to be expressed on the same scale (Figure 3.3). On this basis, the transfer of 16:0, 18:0, 18:1, 18:2, 18:3 and AA followed the same kinetic profile throughout development. In contrast, the transfer of DHA followed a different and unique profile, indicating that DHA was being preferentially transported from the yolk lipids.

As a consequence of this transfer of lipid from the yolk complex, the amount of total lipid in the embryo increased dramatically from day 12 to day 18 and then remained approximately constant over the hatching period (Tables 3.13 and 3.14; Figure 3.4). Whereas PL was the major lipid class in the embryo during the first half of development,

the amount of TG in the embryo increased dramatically from about day 13/14 (Figures 3.5 and 3.6). These results may be interpreted in terms of membrane biogenesis and thus PL accumulation occurring throughout development, with a major deposition of TG in the embryo occurring during the latter part of development, presumably mainly as adipose tissue (see section *3.1.3.*) and intramuscular fat, in parallel with the period of intensive yolk lipid transfer.

The amounts of C16 and C18 fatty acids in the total lipids of the whole embryo, expressed as a percentage of the initial yolk value, showed a very similar pattern of increase from day 11/12 onwards. In contrast, the incorporation of both AA and DHA into the total embryo lipids occurred at a much higher rate, especially during the early phase (day 12-16) of the period of intensive lipid transfer (Figure 3.7). In the case of DHA, this result is compatible with the preferential transfer of DHA from the yolk at this time. The rapid rate of increase of the AA content of the embryo lipids is possibly a result of the conversion of 18:2 to AA by desaturation/elongation in the yolk sac membrane and the embryonic tissues (Noble and Cocchi, 1990).

The results in Figure 3.7 and Table 3.21 also indicate that only about 20% of the mass of the major yolk lipid fatty acids (i.e. the C16 and C18 fatty acids) transferred from the yolk complex actually accumulate in the embryo. This suggests that a large proportion of the fatty acids transferred are utilised for energy by the β-oxidation pathway. However, in the case of AA and DHA, a greater proportion of the transferred fatty acid was recovered in the embryo lipids; presumably this indicates that these long chain PUFA are relatively resistant to β-oxidation, possibly because they are preferentially incorporated into membrane PL, but the probable synthesis of additional AA from 18:2 complicates the interpretation for this fatty acid.

The fatty acid compositions of the major lipid classes of the whole embryo changed in a distinctive fashion during development, particularly with regard to AA and DHA (Tables 3.15 and 3.20). In both the experiments shown, the DHA proportion (% w/w of total fatty acids) in PL was relatively high at the earlier development stages and decreased only slightly at the later stages. Similarly, the AA proportion in PL remained fairly constant throughout development. Relatively high levels of DHA (approx. 10% w/w) were present in total embryo TG at the earlier stages but this proportion decreased dramatically to approx. 1% by day 22. Whole body CE was also characterised by a high proportion of DHA at the earlier stages, but with a much reduced DHA proportion later in development.

The partitioning of DHA between PL and TG of the whole embryo also exhibited some distinctive features (Figure 3.8). Whereas the amount (mg/embryo) of DHA in PL increased steadily from day 12 to 22, the amount of DHA in TG increased dramatically up to day 15 and then remained fairly constant until day 19; during this period, the amount of DHA in TG was approximately twice as much as the amount in

PL. Then, over the hatching period, the amount of DHA in TG dramatically declined. One interpretation of this result, which will be considered in more detail later in the discussion, is that tissue TG may serve as a store of DHA which can be selectively mobilised to provide a supply of this fatty acid for specific developmental functions.

To summarise so far, these studies on the gross transfer of fatty acids from the yolk to the embryo have revealed a number of interesting features:

(i) DHA is *preferentially* transferred from the lipids of the yolk; this is a unique feature appertaining to DHA and is not shown by any other major fatty acid. Previous work (Noble and Cocchi, 1990) has demonstrated that the major proportion of yolk DHA is present as a component of PE and that molecular species of PE which contain DHA tend to preferentially disappear from the yolk from around day 12. Results from two other studies are also compatible with this view (Cherian and Sim, 1992; Lin *et al* 1991). It would therefore appear that there is a specific uptake mechanism to preferentially transfer DHA-containing species of PE from the yolk to the yolk sac membrane. The means by which such a specific process could take place against a background of non-specific phagocytotic uptake of yolk components by the yolk sac membrane (Lambson, 1970) remains to be established.

(ii) As a consequence of (i), DHA preferentially accumulates in the lipids of the whole embryo during the early stages of lipid transfer.

(iii) In the embryo, DHA is incorporated into both PL and TG in a characteristic developmental pattern.

4.2.3. The consequences of differing amounts of initial yolk lipid.

The use of eggs of different sizes and therefore of different initial lipid contents (Table 3.1) provides a simple opportunity to study the effects of different "nutritional" regimes on the development of the embryo. The two experiments described in this section utilised batches of eggs which differed by 1.5- fold in their initial yolk lipid content. Thus, in relative terms, these experiments may be taken to represent conditions of nutritional stringency versus nutritional excess for the embryo.

The fact that the total amount of lipid which accumulated in the embryo was significantly less in the "small" egg experiment (Figure 3.4) is not surprising. More interesting are the effects of initial yolk lipid content on the relative proportions of the major lipid classes in the embryo. A striking feature of the final week of embryogenesis is the deposition of large amounts of TG in the embryo in levels proportional to the initial yolk lipid content (Figure 3.6). Thus the TG accumulation is relatively restricted in the embryos which develop under conditions of comparative nutritional stringency. In contrast, the PL content of the newly-hatched chick was relatively unaffected by the initial amount of yolk lipid (Figure 3.6). It is likely that, at least within ranges of egg

sizes used here, the amount of PL in the embryo is determined by the demands of the inherent developmental programme of membrane biogenesis, whereas the accumulation of TG in the embryo is more dependent on substrate supply.

The results in Table 3.21 suggest that both DHA and AA are relatively resistant to β-oxidation in the embryo, possibly because of preferential incorporation of these fatty acids into membrane PL. This relative resistance was less marked for embryos which developed within the larger eggs, probably due to the greater excess of these fatty acids in the initial yolk (Table 3.1) relative to the comparatively unaltered demands for membrane biogenesis. Given the important roles of AA and DHA in membrane PL (see section *1.4*), it is possible that many biological membranes have an optimal fatty acid composition that is most compatible with normal function. It may be proposed that once this optimal composition has been achieved, any excess AA and DHA supplied from the yolk will be exposed to the possibility of β-oxidation, having being denied "sanctuary" in the membrane PL. Thus development within the larger eggs containing correspondingly greater amounts of AA and DHA in the yolk, will be characterised by the β-oxidation of a greater proportion of the initial AA and DHA content. It is of interest that similar conclusions have been made by Lin *et al* (1991) who compared the fatty acid composition of the whole newly-hatched chick with that of the initial yolk. Firstly, they found that greater proportions of the initial yolk AA and DHA content were recovered in the newly-hatched chick in comparison with the major C16 and C18 fatty acids; thus AA and DHA were relatively resistant to β-oxidation. Secondly, by comparing eggs with differing initial AA and/or DHA contents, they were able to show that this relative resistance to β-oxidation was inversely proportional to the initial amounts of these fatty acids in the yolk. Thus, if an egg contained a high initial amount of DHA, a greater proportion of this DHA would be β-oxidised.

Additional consequences of differences in initial yolk lipid content are illustrated by the results in Table 3.22, in which the amount of TG and PL in the newly-hatched chick were compared with the respective amounts of TG and PL in the initial yolk. In both experiments, generally less than a quarter of the initial amount of yolk TG or PL was recovered in the day-old chick in the form of TG and PL respectively. A reasonable explanation for this finding is that a large proportion of the fatty acyl components of *both* TG and PL are metabolised by β-oxidation. A greater proportion of the initial TG content was recovered as TG in the day-old chick when development occurred within the larger eggs; presumably this reflects the formation of adipose tissue from these fatty acids which are surplus to the energy needs of the embryo. Under the more stringent conditions within the smaller eggs, a greater proportion of the fatty acyl content will be required for energy purposes, leaving less available for adipose tissue formation. In the case of PL, a greater proportion of the initial content was recovered in the day-old chicks which originated from the smaller eggs. The explanation for this is similar to that

advanced in the previous paragraph for individual fatty acids; that is, once the acyl group requirement for membrane biogenesis has been satisfied, the remaining fatty acids will be susceptible to β-oxidation; the amount of fatty acids in excess of requirements for PL synthesis will be lower in the smaller eggs. The above discussion does not, however, imply that fatty acids recovered from embryo TG necessarily originate only from yolk TG, or that tissue PL fatty acids are derived only from yolk PL; in fact it is likely that some "mixing" or cross incorporation occurs. This is evident, for example, from the observation that DHA, which is mainly present in yolk PL, is extensively incorporated into tissue TG (Figure 3.8).

4.2.4. Transfer from the yolk sac membrane to the embryonic circulation.

The concentration of total lipid in the plasma of the embryo increased progressively from day 12 of development resulting in very high plasma lipid levels just prior to hatching at days 18 and 19 (Table 3.23). The plasma lipid concentration then decreased after hatching. These results are similar to those of Yafei and Noble (1990) and are in accordance with the changes in the rate of lipid transfer from the yolk to the embryo (Figure 3.1). At the beginning of the period of rapid lipid transfer (days 12-14), TG was the major plasma lipid component, but the later stages were characterised by decreasing proportions of TG and a predominance of CE. It is not possible to accurately interpret these changes in the absence of data on the proportions of the lipoprotein classes (e.g. very low density lipoprotein (VLDL), VLDL-remnants, low density lipoprotein (LDL), high density lipoprotein (HDL)) and their lipid compositions and, indeed, such studies would be difficult given the small amounts of plasma obtainable from the embryos. However, it may be suggested that the plasma TG is largely present as a component of VLDL secreted by the yolk sac membrane whereas the increase in plasma CE content may partly result from an accumulation of VLDL-remnants in the circulation (i.e. if the rate of remnant production is greater than the rate of remnant uptake by the liver). An additional source of plasma CE could be HDL; i.e. as a result of transfer of surface components including CHOL from VLDL to HDL during LPL action and the subsequent conversion of this CHOL to CE via the lecithin:cholesterol acyltransferase (LCAT) reaction (Brown *et al*, 1981; Dolphin, 1985; Mahley *et al*, 1984; Nilsson *et al*, 1980). A major finding of this study was the presence of high levels of DHA in the TG fraction of the plasma, particularly between days 12-15 when proportions of DHA as high as 14% (w/w of total fatty acids) were detected (Tables 3.26 and 3.30) in this fraction. In contrast, the plasma PL contained only low levels of DHA but much higher (up to 20%) proportions of AA (Tables 3.27 and 3.31). Thus the proportions and distribution of AA and DHA in the plasma are strikingly different from that displayed by their precursors in the yolk. The DHA of the yolk is

mainly present as a component of PL where it comprises 6-8% of fatty acids (Table 3.3) whereas only trace levels of DHA (0.2-0.4%) are detected in yolk TG (Table 3.2). Thus it may be proposed that, following uptake from the yolk into the yolk sac membrane, DHA is hydrolysed from PL and re-esterified into TG. It may be surmised that this newly assembled TG containing high levels of DHA is then incorporated into lipoprotein particles to be secreted from the yolk sac membrane into the embryonic circulation. So far, therefore, DHA transport from the yolk to the target tissues has displayed two unique features: firstly, the preferential uptake of DHA-containing PL species from the yolk into the yolk sac membrane, and, secondly, the translocation of DHA from PL to TG within the yolk sac membrane with the result that DHA is released into the circulation as a component of lipoprotein TG. It should also be noted that the proportion of AA in plasma PL is much higher than in the initial yolk PL (Table 3.3 cf. Tables 3.27 and 3.31). This probably reflects the functioning of the desaturation/elongation system in the yolk sac membrane resulting in the synthesis of additional AA from 18:2 (Noble and Cocchi, 1989, 1990).

A major consequence, therefore of the lipid re-arrangements which occur in the yolk sac membrane is that the long chain PUFA of the *n-6* and *n-3* series show distinct patterns of partitioning between the lipid classes of the secreted lipoproteins. Thus DHA is selectively diverted into lipoprotein TG whereas AA is preferentially incorporated into PL.

The situation described here regarding the processing of yolk lipids within the yolk sac membrane has similarities with the partitioning of fatty acids in the PL and TG of chylomicrons during absorption of lipid by the mammalian intestine. Thus dietary DHA and other *n-3* fatty acids (Anderson and Connor, 1992; Li *et al*, 1992; Nilsson *et al*, 1992) are preferentially incorporated into chylomicron TG whereas AA is preferentially incorporated in chylomicron PL (Nilsson *et al*, 1992; Nilsson and Melin, 1988). For example, Li *et al* (1992) reported that, following oral administration of [^{14}C]DHA to weaning rats, 92% of the radioactivity incorporated into plasma chylomicrons was associated with the TG fraction. This differential partitioning of AA and DHA in both the avian and mammalian systems may result from the substrate specificity of the relevant acyltransferases and probably reflects the outcome of competition between the various fatty acyl species for these reactions (Nilsson and Melin, 1988; Nilsson *et al*, 1987; Rubin and Laposota, 1992).

The subsequent metabolic fate and tissue-specific uptake of the acyl components of the plasma lipid released from the yolk sac membrane will be profoundly influenced by their distribution between the lipid classes in the lipoprotein (Nilsson *et al*, 1992; Nelson and Ackerman, 1988). Furthermore, DHA is ultimately incorporated into the PL fraction of the brain; thus it is striking that this fatty acid initially present in the yolk PL is transferred to the TG fraction in the yolk sac membrane prior delivery to the embryo.

4.2.5. Delivery of lipid moieties to the tissues from the plasma.

The next stage to consider is the fate of the lipoproteins which are released from the yolk sac membrane into the circulation. Of particular interest is the partitioning of specific lipid components of the lipoproteins between different tissues. The results of Speake *et al* (1993) suggest that the tissue-specific expression of LPL is of primary importance in this regard. Thus the high activity of LPL in the embryo's adipose tissue will favour the diversion of a significant proportion of the circulating TG-fatty acids into the adipocytes for storage as TG droplets. Lower, but significant, levels of LPL in the heart and skeletal muscles will enable these tissues to obtain fatty acids, hydrolysed from plasma TG, to be used for β-oxidation and membrane formation. Following the hydrolysis of a large proportion of the lipoproteins' TG content by the action of LPL, the resultant remnant particles consisting mainly of CE are deposited in the liver. As a consequence, very large amounts of CE are deposited in the liver as development proceeds (Shand *et al*, 1994).

The action of LPL on the plasma lipoproteins not only determines the amount of fatty acid supplied to specific organs but also to some extent, influences the composition of the fatty acids which are delivered (Speake *et al*, 1993). A number of studies have indicated that TG species which contain C20 and C22 PUFA exhibit a degree of resistance to LPL action (Melin *et al*, 1991, Nilsson *et al*, 1987; Ridgeway and Dolphin, 1984). These studies showed that the FFA released from plasma chylomicrons by LPL action contained a lower proportion of C20 and C22 PUFA than were present initially in the chylomicron TG. Also, the TG remaining in the resultant remnant particles were enriched in polyenoic C20 and C22 acyl groups. Speake *et al* (1993) proposed that this substrate specificity of LPL could explain some of the striking features of tissue lipid fatty acid composition in the chick embryo. Thus the high proportion of DHA in plasma TG (approx. 14%, Tables 3.26 and 3.30) is only partially reflected in the composition of adipose TG where DHA forms about 6% of fatty acids (Table 3.37). However, it may be proposed that the residual TG of the lipoprotein remnant will be further enriched in DHA; since the remnant are taken up by the liver, this would explain the very high proportions of DHA in liver TG (approx. 18%; Table 3.40). [The above values for the % of DHA in TG are based on the results at days 12-14].

The above discussion has described the likely mechanisms by which lipids may be delivered to adipose tissue, heart, muscle and liver of the embryo. The key question is the mechanism by which the developing brain obtains its lipid requirements. Thus far, two mechanisms of tissue-specific lipid uptake have been described: (i) via the localised action of LPL situated in the capillaries permeating specific tissues; (ii) via the (receptor-mediated?) uptake of lipoprotein remnants. However, LPL activity was not detected in the chick embryo brain at any stage (Speake *et al*, 1993). As regards the second

possibility, recent studies have provided evidence that cells of the mammalian central nervous system express receptors for LDL, HDL and the lipoprotein remnants (Handelman *et al*, 1992; Pitas *et al*, 1987). The expression of such receptors in the brain of the chick embryo, enabling neural uptake of lipoprotein remnants, would provide an extremely efficient means of supplying the developing nervous system with DHA, since, as proposed above, it is likely that such remnants are especially enriched in DHA. Such a mechanism would also provide a supply of cholesterol for myelination, and it is of interest that the receptor-mediated uptake of lipoproteins by foetal rabbit dorsal root ganglion cells was found to promote neurite growth, extension and branching (Handelman *et al*, 1992). However, a recent study from this Department indicating that large amounts of vitamin E accumulate in the chick embryo's liver, apparently as a result of remnant uptake, whereas only extremely low levels of this vitamin are detected in the brain (Gaál *et al*, 1995) suggest that uptake of lipoprotein remnants by neural tissues may not be a significant process in this system. Also, it appears that the brain of the chick embryo obtains its myelin cholesterol requirements by *de novo* synthesis of cholesterol as opposed to uptake from plasma lipoproteins (Noble and Cocchi, 1990).

It is possible, therefore, that the developing chick brain does not directly obtain its PUFA requirements from the yolk sac-derived lipoproteins or their remnants. Although the proportion of DHA in adipose TG is somewhat lower than in the circulating lipoprotein, the absolute amount of DHA deposited in the tissue (i.e. mg DHA/depot) is relatively very high (Figure 3.13) due to the large amount of lipid in this tissue. An opposite, but equally interesting, situation appertains to the liver TG; the absolute amount of DHA in liver TG (mg DHA/liver) is much lower than in the adipose tissue but, DHA forms a very large proportion of total TG fatty acids in the liver (Table 3.40 and Figure 3.17). These observations raise the possibility that adipose tissue and/or liver may somehow mediate the delivery of DHA to the brain. The following sections discuss the changes in the fatty acid composition of the lipid of adipose tissue (section *4.2.6.*) and liver (section *4.2.7.*) during development in order to provide a background for such speculations. The fatty acid compositions of the lipid of the heart (section *4.2.8*) and finally the brain itself (section *4.2.9.*) are also considered.

4.2.6. Fatty acid profile of adipose tissue TG during development.

The accumulation of TG in the subcutaneous adipose depot of the embryo between days 12 and 19 was dependent on the amount of initial yolk lipid (Figure 3.10) and on the amount of lipid transferred from the yolk at any given time (Figure 3.11). However, the linear relationship between yolk lipid transfer and adipose tissue growth completely broke down over the hatching period (Figure 3.11). Thus although

approximately 37% of the total lipid transfer occurred between days 19 and 22, there was no further growth of adipose tissue during this period. This may reflect a decrease in LPL activity in the tissue at this time (Speake *et al*, 1993) coupled with an increase in the sensitivity of the adipocytes to lipolytic agonists (Langslow, 1972).

Whereas the amounts (mg fatty acid in TG/depot) of the major C16 and C18 fatty acids in the tissue TG changed essentially in parallel with the profile of TG accumulation in the depot (i.e. a continuous increase between days 12 and 19, then remaining constant or slightly decreasing over the hatching period, Figure 3.12), a totally different and unique pattern of change was found for DHA (Figure 3.13). Instead of increasing continuously until day 19, the amount of DHA in the depot reached a plateau level by day 16 with little or no further increase prior to hatching. More dramatically, the amount of DHA in adipose TG decreased precipitously over the hatching period.

Firstly, it is clear that adipose TG in the embryo represents a substantial store of DHA. In fact, between days 14 and 19, considerably more DHA was present in the lateral thoracic adipose depot alone than in the total brain PL (Figure 3.13 cf Figure 3.14). Secondly, the dramatic and specific decrease in the DHA content of the adipose depot during hatching suggests that this fatty acid may be preferentially mobilised from the tissue TG. Additionally, the relatively constant amount of DHA in the depot from days 16 to 19 may represent a balance between incorporation of the fatty acid from plasma lipoprotein via LPL action and this specific mobilisation of DHA from the tissue.

These results are consistent with the possibility that adipose tissue may function as a mediator of DHA transport from the yolk to the brain. The preferential mobilisation of DHA from adipose TG will result in this fatty acid being released into the circulation in the FFA form, presumably bound to serum albumin. As mentioned in the Literature review (section *1.4.5.*) there is considerable evidence that the brain can take up PUFA, in the FFA form, from the plasma. These points are considered in more detail later in this discussion (section *4.5.2.*).

4.2.7. Fatty acid composition of the liver lipids during development.

The lipid composition of the liver during the latter half of avian development is largely a consequence of the uptake of lipoprotein remnants which consist mainly of CE (predominantly as cholesteryl oleate) with some residual TG which contains high levels of DHA and also PL enriched in AA (Noble and Cocchi, 1990; Shand *et al*, 1994). The results in Tables 3.39 to 3.43 and Figure 3.15 are clearly consistent with the previous studies on liver lipid composition in the chick embryo (Noble and Cocchi, 1990).

The results shown in Figure 3.16 indicate that significant amounts of both AA and DHA accumulate in the total lipids of the liver, reaching maximal levels just prior to

hatching. Thus the liver may also be considered as a "store" of DHA in the embryo. Clear evidence that the liver also functions as a mediator of DHA delivery to the brain (i.e. by mobilising the stored DHA) is not directly forthcoming from this data. However, these results do suggest that during the hatching period DHA may be transferred from TG to PL in the tissue. Firstly, between days 19 and 22, the proportion (% w/w of total fatty acid) of DHA in liver TG decreased sharply in parallel with a marked increase in the proportion of DHA in PL (Tables 3.40 and 3.41). Secondly, Figure 3.17 illustrates that the amount (mg DHA in lipid class/liver) of DHA in liver TG decreases dramatically over the hatching period in parallel with an equivalent increase in the amount of this PUFA present in the PL fraction. Furthermore, this transfer between TG and PL appears to be specific for DHA and is not exhibited by any other fatty acid; another unique feature of DHA transport.

Investigations into the mechanism of DHA transport to the brain and retina of neonatal rodents have suggested a major role for the liver in the mediation of this process (Li *et al*, 1992; Scott and Bazan, 1989). In these studies, DHA was apparently transferred from TG to PL in the liver, and was secreted in this form as a component of VLDL for subsequent delivery to the target tissues. The observation in the present study regarding the selective increase of DHA content of PL in the liver of the chick during hatching (Figure 3.17) may reflect a switch from the putative adipose tissue mediated mechanisms of fatty acid delivery suggested above (section *4.2.5.* and Figure 4.2) to a liver mediated process, similar to that suggested for neonatal mammals.

4.2.8. Fatty acid composition of the heart lipids during development.

The heart can not be considered as a major site of lipid accumulation during embryonic development (Table 3.44). PL is the major lipid class in this tissue and is characterised by a high content of AA (Table 3.47). The TG of the heart contains a significant proportion of DHA, particularly at the beginning of the final week of embryonic life (Table 3.45). This presumably results from the action of heart LPL on the DHA-rich TG of the plasma lipoproteins (Speake *et al*, 1993). In terms of the absolute amount of DHA in the heart, less than 20 μg of DHA were present per heart in the TG fraction with about 5-times this amount per heart present as a component of PL (Figure 3.18). These amounts remained approximately constant throughout the final week of embryogenesis.

Thus, unlike adipose tissue and liver, the heart is not, in quantitative terms, a major destination for the DHA transferred from the yolk; nor is there any evidence that this tissue plays a role in mediating DHA delivery to the brain. However, the PL of the heart does display a characteristic composition and important roles for both AA and DHA in heart function can not be ruled out.

4.2.9. Fatty acid composition of brain PL during development

The period of rapid lipid transfer from the yolk to the embryo (i.e. the last week of development is also a period of rapid brain growth. For example, in the experiment described in Table 3.50, the brain fresh weight increased by 7.2-fold and the amount of total lipid per brain increased by 11.3-fold between days 12 and 22. PL was the major lipid class in this tissue throughout embryogenesis with CHOL composing most of the remainder. For the experiments described in Tables 3.49 and 3.51, the proportions of PL and CHOL remained fairly constant prior to hatching. However, the experiment in Table 3.49 was continued for 4 days after hatching, and at this stage a decrease in the proportion of PL and an increase in the proportion of CHOL was observed.

The fatty acid composition of brain PL, measured in 3 separate experiments, did not show any major changes between days 11 and 25 (Tables 3.49, 3.50 and 3.51). In two of the experiments, some decrease in the proportion of 16:0 did occur, particularly after hatching. However, a key feature was that the proportions of AA and DHA remained remarkably consistent throughout the second half of embryonic development and during the first few days after hatching. At most stages, DHA was the second most abundant fatty acid (after 16:0) comprising up to 22% (w/w) of the total, with AA comprising 8-10%.

The development of the central nervous system of the chick begins as early as the first week of incubation with the formation of the neural plate and by day 4 most of the basic forebrain structures have formed (Romanoff, 1960; Rogers, 1995). Prior to the period of rapid lipid transfer, brain development at the cellular level is mainly characterised by rapid cell proliferation with the peak rate of neurogenesis occurring at day 8 (Freeman and Vince, 1974; Rogers, 1995; Sobue and Nakajima, 1978). The next phase of brain development is characterised by neurite outgrowth, axonal extension and dendritic branching, ultimately resulting in synapse formation with the peak rate of synaptogenesis occurring at day 15 (Rogers, 1995; Sedlacek, 1972). The maturation of the synaptic connections and the formation of myelin sheaths then continues into post-hatch life (Rostas, 1991). The developmental period studied in the present work (essentially from day 12 to day 22), therefore, encompasses the stages of neurite outgrowth and synaptogenesis which, in developing mammals, have been shown to be characterised by the accumulation of DHA in PL of the neuronal membranes (Dobbing and Sands, 1979; Neuringer *et al*, 1988). The DHA content of myelin PL of the chick embryo brain is very low (Alejandre *et al*, 1984) consistent with the view that DHA is incorporated largely into the neuronal membranes. The fatty acid compositions of the brain PL reported here are consistent with previous studies carried out on the brain of the newly-hatched chick (Anderson *et al*, 1989, 1990; Cherian and Sim, 1992; Lin *et al*, 1991). Rizzo *et al* (1995) have made a more detailed study of brain PL composition

during chick embryo development. They reported minor differences in the AA and DHA levels between different regions (i.e. cerebral hemispheres, optic lobes, cerebellum and brainstem) and also between the left and right hemispheres.

The absolute amount of DHA in the brain PL (mg DHA/brain) increased in a continuous fashion throughout the second half of the embryonic period (Figure 3.19). Thus the brain has an ongoing requirement for an adequate supply of DHA during this period. The amounts of 16:0 and AA (Figure 3.19), and indeed of all the major fatty acids, increased in a fashion parallel to the changes in DHA content, reflecting the relatively unchanging proportions of the major fatty acids (Tables 3.49 to 3.51) during development.

4.2.10.Summary of this section

This study of the amounts and relative proportions of the major fatty acids in the lipids of the yolk complex, whole embryo, blood plasma and key tissues throughout development has revealed one striking feature: the unique behaviour of DHA at almost every stage of the transport process. These characteristics are reflected in unique patterns of distribution of DHA between tissues and between lipid classes and are generally not displayed by the other major fatty acids. These aspects of DHA transport are listed below:

(i) DHA is preferentially taken up from the yolk by the yolk sac membrane.

(ii) Within the yolk sac membrane, it appears that DHA is selectively translocated from yolk PL into lipoprotein TG. Thus DHA enters the embryonic circulation as a component of plasma TG. The chief consequence of this will be that DHA will become susceptible to hydrolysis from the lipoprotein by the action of LPL, since TG, not PL, is the preferred substrate for this enzyme (Olivecrona and Bengtsson-Olivecrona, 1987).

(iii) As a consequence of the especially high LPL activity expressed in the adipose tissue, a large amount of DHA is incorporated into adipose TG.

(iv) As a consequence of the substrate specificity of LPL, the residual TG of the lipoprotein remnant will be especially enriched with DHA; as a result of remnant uptake by the liver, this tissue will accumulate TG which, although low in amount compared with adipose tissue, contains a very high proportion of DHA.

(v) The changes in the amount of DHA present in adipose TG during development suggest that DHA may be *preferentially mobilised* from this tissue. This would result in the release of DHA as the FFA into the circulation; it is suggested that, in agreement with the work of other authors (Dhopeshwarkar and Mead, 1973; Anderson and Connor, 1988), this DHA may be taken up by the brain from the plasma in the FFA form. Thus it is suggested that adipose tissue may play a key role in mediating DHA transport to the brain.

(vi) In the liver, over the hatching period, DHA is apparently selectively translocated from TG to PL. The significance of this is not clear but it may represent the early stages of a role for the liver as a mediator of DHA supply to the brain in the neonatal bird.
(vii) Both DHA and AA appear to be relatively resistant to β-oxidation in the embryo. Similar findings for the chick embryo have also been reported by Lin *et al* (1991). It is also of interest that differential rates of oxidation of saturated and polyunsaturated fatty acids were also observed in the developing rat (Leyton *et al*, 1987).

The fact that DHA, which in quantitative terms is a relatively minor fatty acid of the initial yolk lipids, exhibits such a range of unique and specialised features during its transport through the embryonic system, suggests strongly that this fatty acid is of crucial importance for the development of certain embryonic structures and functions. This view is consistent with the evidence for the importance of DHA, particularly in the development of the neural tissues, as described in section *1.4* and *4.1*. It is perhaps somewhat surprising, therefore, that the one tissue in this study which, at least from the compositional data, does not display any unique features regarding DHA incorporation, is the brain itself. The proportion of DHA in brain PL remained approximately constant throughout the period studied (Tables 3.49 to 3.51) and, as a consequence, the amount of DHA in the tissue (mg DHA/brain) increased in parallel with the other major fatty acids (Figure 3.19). However, in order to directly assess the question of preferential uptake, a knowledge of the acyl composition of the pool of fatty acids which serves as a precursor for uptake into the brain (e.g. the plasma FFA fraction) plus studies on the differential rates of uptake and incorporation of the various fatty acids into brain PL using radioactive tracer studies, are also required. It is of interest that plasma FFA fraction in this study contained very high proportions of DHA (up to 17%, w/w; Tables 3.29 and 3.33), at least between days 12 and 16 of development. This result is consistent with the view that DHA may be being preferentially mobilised from adipose TG into the plasma FFA fraction and would also seem to obviate the need for any preferential uptake of DHA by the brain at this stage.

The selective uptake of a particular fatty acid by the brain could involve differential transfer across the blood-brain barrier (Alberghina *et al*, 1993, 1994; Anderson *et al*, 1993; Spector, 1988), specific uptake mechanisms into the neurons based on the specificity of fatty acid binding proteins (Abumrad *et al*, 1989; Schoentgen *et al*, 1989) or the fatty acyl specificity of the acyltransferases in the brain cells responsible for the esterification of fatty acids into PL (Esko and Raetz, 1983). In mammals, there is considerable evidence for the preferential uptake of DHA by the neural tissues, as studied *in vivo* and *in vitro*. For example, Anderson and Connor (1988) found that DHA was taken up into the brain more rapidly than were other fatty acids following injection of the radioactive tracer FFA into functionally hepatectomised rats, whereas Sarda *et al* (1986) reported the selective incorporation of injected DHA

into different brain regions of the rat. In addition, Onuma *et al* (1984) reported the selective incorporation of DHA into brain lipids following intracranial injection. Furthermore, *in vitro* studies have demonstrated the preferential uptake of DHA into lipids of the retina of monkeys and humans (Rodriguez *et al*, 1990). In contrast, isolated brain cells of the juvenile turbot did not show any preferential uptake of DHA (Tocher *et al*, 1992). The results of the present study may be taken to suggest that the chick embryo system is more similar to the fish than the mammal in terms of the apparent lack of preferential DHA uptake by the brain. However, the evidence is at present circumstantial, based solely on the compositional data; more direct studies are therefore needed to test this view.

The question arises as to the need for the preferential transfer from the yolk to the embryo at the beginning of the period of rapid lipid transfer (approx. day 12 to day 15; Figure 3.3) since the proportion of DHA in brain PL does not increase during this period (Tables 3.49 to 3.51) and the rate of increase in the absolute amount (mg DHA/brain) is actually slightly lower than that at the later stages (Figure 3.13). Also, of course, preferential transfer at an early stage means that there is less DHA available for transfer at the later stages. It should be noted that this preferential transfer of DHA has a profound effect on the fatty acid composition of the embryo's lipids, particularly of the TG fraction. Thus, at around day 12 high proportions of DHA are present in the TG fraction of the whole embryo (Tables 3.15 and 3.18), the plasma (Tables 3.26 and 3.30), adipose tissue (Table 3.37), liver (Table 3.40) and heart (Table 3.45). A common feature of all these examples is that the proportion of DHA in the TG subsequently decreases as development proceeds, due to the dilution of the DHA by other incoming fatty acids. Thus, by the time of hatching, the proportion of DHA in these TG fractions is generally quite low. In contrast, the proportion of DHA in the PL fractions of the whole embryo (Tables 3.16 and 3.19), plasma (Tables 3.27 and 3.31), liver (Table 3.41) and brain (Tables 3.49 to 3.51) either decreases only slightly or remains fairly constant as development proceeds.

One explanation for the selective transfer of DHA to the embryo could be that this process would allow the transport of this fatty acid to be partially uncoupled from the gross energy needs of the embryo. This may be necessary in order to enable the supply of adequate levels of DHA to the brain prior to the period when lipid utilisation for energy purposes is maximal. An alternative explanation is that preferential transfer of DHA from the yolk promotes the accumulation of DHA in adipose TG at an early stage; in fact from the time of the initial formation of the tissue. Thus the adipose tissue becomes "pre-loaded" with DHA which is subsequently preferentially mobilised to supply the needs of the developing brain.

4.3.BRAIN PHOSPHOLIPID CLASSES AND THEIR FATTY ACID COMPOSITION.

4.3.1.Significance of the compositional data for the PL classes.

The HPLC method devised by Christie (1985) was found to be very suitable for the isolation of the major PL fractions (Figure 3.20) in a highly purified form as assessed by HPTLC. As regards the PUFA distribution between the different PL classes of the brain, a consistent pattern was observed in both the chicken and the duck (Tables 3.52 to 3.60). Thus, although PC was the most abundant class of PL in the embryonic brain, it contained only minor proportions of both DHA and AA. The second most abundant PL class, PE, was the major molecular site of PUFA accumulation in the brain, containing up to 40% (w/w) DHA with lower (around 12%) proportions of AA. The PUFA content of PS was similar to that of PE, but with a lower proportion of AA. In contrast, the quantitatively minor class, PI, contained up to 45% AA but only low levels of DHA. These results are very similar to those reported for the developing mammalian brain (Green and Yavin, 1993; Neuringer *et al*, 1988).

A number of studies have investigated the incorporation of radioactively-labelled fatty acids into different PL classes of the neural tissues during mammalian development. Anderson and Connor (1988) noted that whereas labelled 16:0, 18:2 and 18:3 were mainly incorporated into PC, labelled DHA was preferentially recovered in the PE fraction, following injection into young rat. Similarly, Gazzah *et al* (1995) also demonstrated that labelled DHA, injected into young rats, was specifically incorporated into the PE fraction of the brain. However, following intracranial injection of DHA into neonatal rats, the label was approximately equally distributed between PC and PE (Onuma *et al*, 1984), suggesting that the route of injection may affect the subsequent partitioning. Delton *et al*, (1995) reported that labelled AA and DHA were differentially incorporated into the lipid classes of cultured pineal cells, with AA largely partitioning into PC whereas more DHA was recovered in PE. These results suggest that the distribution of PUFA between the PL classes may differ in particular brain regions. This same paper (Delton *et al*, 1995) also investigated the effect of stimulation by nor-adrenaline on the distribution and release of PUFA by the pineal cells and provided evidence that both AA and DHA may be involved in the signal transduction process leading to the release of the pineal hormone melatonin. The studies of Tocher *et al* (1992) using brain cells isolated from the developing turbot indicated that labelled DHA was incorporated into both PC and PE in this system.

It is well established that PI (in the PI-4'5-biphosphate form) has a major role in the signal transduction pathways associated with many aspects of brain development and function (Furuichi and Mikoshiba, 1995). Furthermore, the activation of specific

isoforms of protein kinase C via the PI pathway is associated with the differentiation of astrocytes in the cerebrum and cerebellum of the chick embryo brain (Mangoura *et al*, 1995a, 1995b). The high level of AA present in the PI fraction of the brain may be of functional significance since the release of this fatty acid from PI and other PL classes following appropriate stimulation and phospholipase A2 activation results in a range of regulatory effects on brain cell function (L'hirondel *et al*, 1995; Peterson *et al*, 1995; Williams *et al*, 1995).

Since most of the brain's DHA is present in PE, it would appear that this lipid class has important roles in those aspects of brain development associated with a requirement for DHA. For example, it may be proposed that PE species containing DHA exhibit some, as yet unspecified, role in the neurite extension process. A recent study by Calderon *et al* (1995) has indicated a preferential sorting of PL and glycolipid species to the membrane of the growing neurite during neuronal differentiation but unfortunately the fatty acids compositions of these species were not measured.

4.3.2. Comparison between the chicken and the duck

The amounts of AA and DHA in the initial yolk of the duck eggs were very different from those in the chicken eggs (Table 3.57). Thus the chicken eggs contained nearly 6-times more DHA (in terms of mg DHA/yolk) than the duck eggs although the latter were more enriched in AA. To some extent, these differences were reflected in the subsequent fatty acid compositions of the PL classes. For example, just prior to hatching (day 18/19), the proportion of DHA in the PE fraction was more than twice as high in the chicken than in the duck (Table 3.54 cf Table 3.59). However, the extent of the differences in brain fatty acid composition was much less than in the fatty acid contents of the initial yolks, between the two species. This suggests that the transfer of DHA from the yolk to the brain was more efficient in the duck than in the chicken, thus partly compensating for the low level of this fatty acid in the duck eggs. An extra source of DHA for the duck embryo could be 18:3, as the duck yolk had more than 2-times more 18:3 than the chicken egg. This suggests that elongation/desaturation systems may provide a source of DHA in the duck embryo.

4.4-PREFERENTIAL MOBILISATION OF LONG CHAIN PUFA DURING *IN VITRO* INCUBATIONS OF ADIPOSE TISSUE PIECES.

There is a great deal of information available relating to the mechanism and regulation of the mobilisation of fatty acids from adipose tissue TG and the release of these fatty acids into the circulation (Fain and Garcia-Sainz, 1983; Newsholme and

Start, 1973; Vernon and Clegg, 1984). In brief, the binding of lipolytic hormones such as adrenaline and glucagon to specific receptors on the plasma membrane of the adipocyte triggers a cascade of events in the cell which result in the activation of Hormone-sensitive lipase (HSL). Hormone binding induces a conformational change in the receptor causing it to interact, via an intermediary G protein, with the enzyme adenyl cyclase. The subsequent activation of this enzyme results in the production of cyclic AMP which in term activates protein kinase A. This phosphorylates and activates HSL which in turn hydrolyses fatty acyl groups from adipose TG. Complete hydrolysis of TG to release glycerol and FFA from the cell requires the participation of diacylglycerol- and monoacylglycerol lipases but it is HSL which catalyses the rate limiting step. Mobilisation is inhibited by insulin and glucose which promote the re-esterification of the released FFA; and by adenosine which de-activates adenyl cyclase via an inhibitor G protein. Some of these aspects are illustrated in Figure 4.1.

Although many of the above details were elucidated using adipocytes isolated from the rat, adipose tissue from the adult chicken has also been used extensively in this work (Belfrage *et al*, 1984). The sensitivity of adipocytes to glucagon-stimulated mobilisation has been studied in chickens at various post-natal ages (Oscar, 1991, 1992, 1995). Changes in the sensitivity of chicken adipocytes to various lipolytic agonists during embryonic and early post-hatch life have been reported by Langslow (1972).

Almost all the investigations on the mobilisation of adipose lipid in both mammalian and avian systems have been concerned with the non-specific release of fatty acids from the fat cells in order to provide energy for other tissues. Relatively few studies have focused on the composition of the mobilised fatty acids. A series of reports in the 1960's on the release of fatty acids from adipose tissue both *in vivo* and *in vitro* failed to provide any evidence for differential mobilisation of the various fatty acids (Nakamura *et al*, 1966; Rothlin *et al*, 1962; Spitzer *et al*, 1966; Stein and Stein, 1962). However, also in the 1960's, Meinertz (1963) demonstrated that 18:1 was selectively retained in adipose TG during *in vitro* incubation whereas Hollenberg and Angel (1963) showed that unsaturated fatty acids were mobilised to a greater extent than their saturated counterparts. It was not until the 1990's that these studies were extended to consider the mobilisation of the C20 and C22 PUFA from adipose TG. Gavino and Gavino (1992) reported that cultivated pre-adipocytes challenged with nor-adrenaline preferentially released the *n-6* and *n-3* PUFA and retained the saturated fatty acids. In the same paper, these authors also showed that rat epididymal fat pads perfused with nor-adrenaline preferentially released AA and 18:3 but tended to retain 18:1 and 18:2. Perhaps the most detailed study in this area is that of Raclot and Groscolas (1993) who evaluated the mobilisation of 52 fatty acids from adipocytes isolated from fish oil-fed rats and incubated with nor-adrenaline. In particular, they found that AA and EPA were preferentially mobilised from the TG of the cells and the main general conclusion from

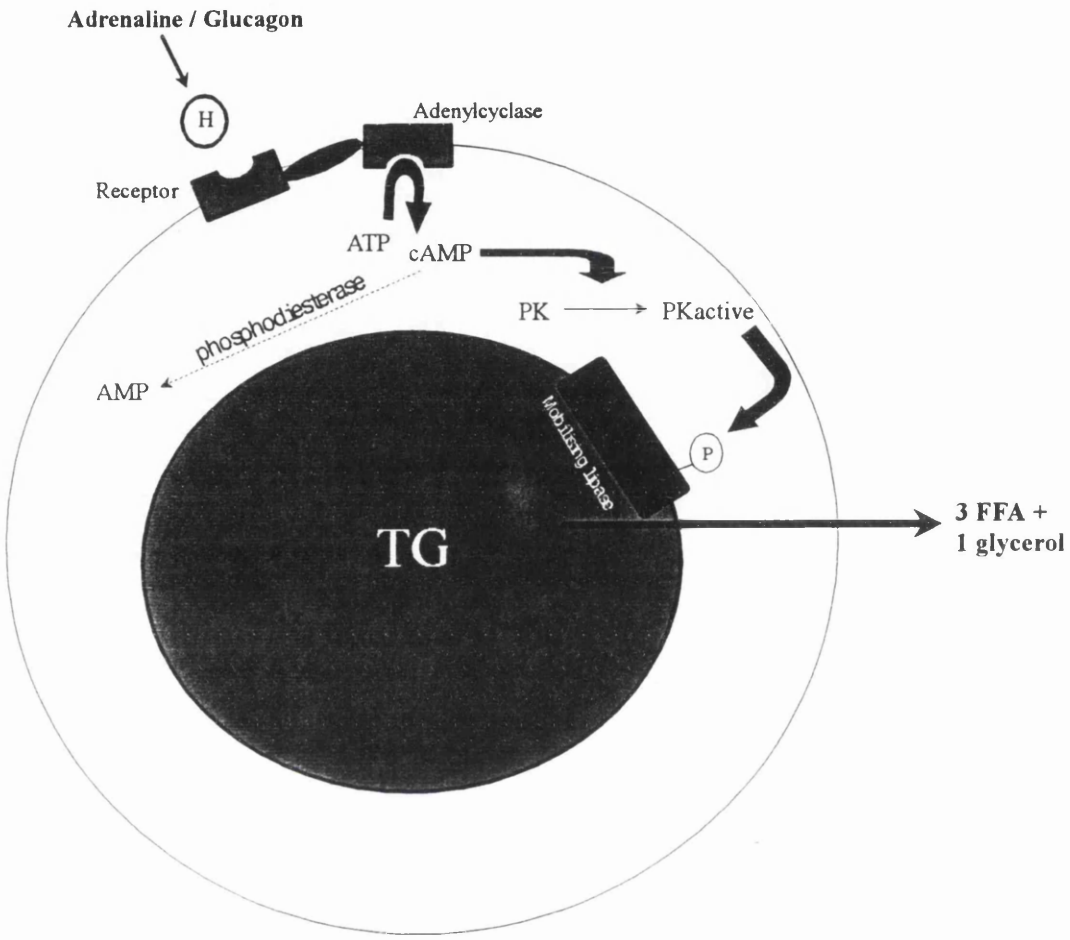
this was that mobilisation increased with increasing unsaturation but decreased with increasing chain length. Crucially, DHA was not selectively mobilised in this study, the effect of high unsaturation being cancelled out by the effect of long chain length.

In the present thesis, the results in Figure 3.13 illustrating the changes in the amount of DHA in adipose TG of the chick embryo at various stages of development suggest that DHA may be preferentially mobilised from the tissue. In order to test this possibility, a series of experiments were carried out in which adipose tissue pieces were incubated *in vitro* and the composition of the FFA released into the incubation medium was compared with that of the tissue TG. The results (Tables 3.61 to 3.70) agreed with those of Raclot and Groscolas (1993) in that the PUFA 18:3, 20:4, 20:5 and 22:5 were found to be preferentially released from the tissue into the medium whereas the major fatty acids of the tissue TG, 16:0 and 18:1, were selectively retained. However, in contrast to Raclot and Groscolas (1993), DHA was also found to be significantly and consistently preferentially mobilised. This preferential mobilisation of DHA occurred at all developmental stages, during both 1h and 24h incubations, in the absence and presence of glucagon and in experiments using adipose tissue from the embryonic pheasant as well as from the chicken.

The difference between the results with adipose tissue from the embryonic chicken as opposed to the adult rat may reflect a specific feature of embryonic life with the need to supply DHA, as the plasma FFA, to the developing neural tissues. This implies that the substrate specificity of HSL may differ according to the needs of particular stages of development. Gavino and Gavino (1992) showed that partially purified preparations of HSL from adipose tissue preferentially cleaved 18:3, but not 18:1, from an artificial TG substrate, suggesting that differential mobilisation was due to the substrate specificity of the enzyme. On the other hand, Raclot and Groscolas (1993) suggested that the more polar TG species (i.e. those containing short-chain unsaturated fatty acids) would be preferentially localised at the periphery of the TG droplet and thus more accessible to the enzyme. Therefore properties of both the enzyme and the substrate have been involved to explain preferential mobilisation.

In summary, the results from the *in vitro* studies support the supposition that DHA (and other PUFA) may be preferentially mobilised from adipose tissue stores, thus releasing the fatty acid into the plasma in a form suitable for uptake by the brain.

Figure 4.1: Mechanism of fatty acid release from adipose TG. Lypolitic agents, such as the hormones adrenaline and glucagon bind to the membrane receptors of the adipocyte. This start a cascade reaction in the cell: adenylcyclase transform ATP to cAMP which activates protein kinase A. The active form of protein kinase A phosphorylates and activates the mobilising lipase (i.e. HSL), thus triggering fatty acid hydrolysis from adipose TG. Mobilising lipase hydrolyses fatty acid in sn-1 or sn-3 positions. Complete hydrolysis will be ensured by di- and mono-acylglycerol lipases. Three molecules of free fatty acids which become bound to albumin and one molecule of glycerol are released into the blood circulation. Phosphodiesterase is antagonistic to this process by removing cAMP.


Adrenaline / Glucagon
H
Adenylcyclase
Receptor
ATP
cAMP
PK
PKactive
phosphodiesterase
AMP
P
TG
3 FFA +
1 glycerol

4.5 COMPARISON OF AVIAN AND MAMMALIAN SYSTEMS

4.5.1.Studies in mammalian systems

There is currently no clear consensus in the literature as to the means by which DHA is supplied to the developing mammalian brain. The investigations of Scott and Bazan (1989) and Li *et al* (1992) in the postnatal rat suggest that DHA from dietary sources is not transported directly from the intestine to the neural tissues but instead the liver plays a major role in mediating this process. Their results suggest that DHA is incorporated into liver PL which is then secreted, presumably as VLDL, and delivered in this form to the brain. The question still outstanding from these works is the mechanism of uptake by the brain since the studies of Dhopeshwarkar and Mead (1973) and Rapoport (1995) indicated that PUFA can not be taken into the brain whilst in the PL form. Also, even though the neonatal mammalian brain may transiently express LPL activity (Ben-Zeev *et al*, 1990), PL is a very poor substrate for this enzyme. The study of Burdge and Postle (1994) has focused on the increased synthesis in the maternal liver of PL species containing DHA and the secretion of these species into the maternal plasma during pregnancy. However, the question arises as to the means by which the DHA of these PL species could be transported across the placenta since it appears that only the FFA form is amenable to transplacental uptake (Crawford *et al*, 1981; Noble and Cocchi, 1989, Kuhn and Crawford, 1986). An alternative mechanism of DHA delivery to the brain was suggested by the studies of Dhopeshwarkar and Mead (1973), Anderson and Connor (1988) and Rapoport (1995) which claimed that the main or even sole, source of PUFA for the brain are plasma FFA bound to albumen or α-fetoprotein. Thiès *et al* (1992, 1994) provided evidence that, although FFA bound to plasma albumen are taken up by the brain, a preferred source may be lyso-PC bound to albumin.

Although there may be some variation between species it is generally believed that provision of the developing fetus and neonate with "pre-formed" DHA is a far more efficient means of enhancing the PUFA status of the developing brain than by supplying 18:3 as a DHA precursor (Crawford, 1993). For example, Sinclair (1975) demonstrated that pre-formed C20-PUFA are incorporated into the developing rat brain at a 10-fold greater efficiency when compared to their C18 precursors. In the human, the conversion of 18:3 to DHA is very low in the fetus (Crawford *et al*, 1981) and apparently non-existent in the placenta (Kuhn and Crawford, 1986). The synthesis of C20-PUFA from their C18 precursors also appears to be very low in the human neonate (Cockburn, 1994). However, the postnatal rat liver does have some ability to synthesise DHA from 18:3 (Nouvelot *et al*, 1986; Scott and Bazan, 1989). Voss *et al* (1991) reported that the rat liver synthesises DHA via the elongation/desaturation of 18:3 to 24:6 which is then retro-converted to DHA. Pawlosky *et al* (1994) reported a highly

24:6 which is then retro-converted to DHA. Pawlosky *et al* (1994) reported a highly specific pathway of elongation/desaturation in the feline where 18:3 is metabolised to EPA in the liver and then EPA is transferred to the brain for conversion to DHA. There is also some evidence that the capillary endothelium of the brain can synthesised DHA from 18:3 via elongation/desaturation and retroconversion steps (Moore *et al*, 1993). Recent work has shown that neurons themselves can not synthesise DHA but that they can avidly take up DHA which has been synthesised and released from neighbouring glial cells (Moore *et al*, 1991).

4.5.2.Mechanism of DHA delivery to the brain in the chick embryo.

The results of the present study do not allow a definitive description of the mechanism by which DHA is transferred from the yolk to the brain to be made. However, a hypothesis can be suggested which is consistent with these results and with mechanisms which are known to operate in other systems.

One of the major findings of the study is that, whereas DHA is present both at its "starting point" in the yolk and at its final destination in the brain in the form of PL, it is apparently transported into the embryonic circulation in the plasma TG. Presumably such a drastic, unique and almost total translocation of DHA between these two lipid classes, apparently occurring in the yolk sac membrane (Figure 4.2), must be of major relevance to the mechanism of transport of DHA to the neural tissues. A major consequence of this translocation will be that the DHA content of the plasma lipoproteins will become susceptible to hydrolysis by LPL, since TG and not PL is the primary substrate for this enzyme (Olivecrona and Bengtsson-Olivecrona, 1987). However, the brain of the chick embryo has no detectable LPL activity (Speake *et al*, 1993); thus any uptake of DHA by the brain following the localised hydrolysis of this fatty acid from plasma TG is unlikely. On the other hand, the adipose tissue of the embryo contains very high levels of LPL activity (Speake *et al*, 1993); as a result, relatively large amounts of DHA are incorporated initially into adipose TG. The evidence, both from the changes in the amount of DHA in adipose TG *in vivo* and from the mobilisation of DHA from tissue pieces *in vitro*, suggests that this fatty acid is preferentially mobilised from the tissue TG and released into the circulation as the FFA. It is suggested that the brain is able to take up DHA, in the FFA form, from the plasma in agreement with Dhopeshwarkar and Mead (1973), Anderson and Connor (1988) and Rapoport (1995). Thus it is proposed that adipose tissue mediates the transfer of DHA from the yolk to the brain. It is to be noted that the major period of DHA accretion in the brain, from day 12 to after hatching, is co-incident with the period of the initial formation of adipose tissue.

An additional possible mechanism, also involving events occurring at the adipose depot, may be suggested. Several studies in mammalian systems have indicated that, although the primary result of LPL action on plasma TG is to release FFA for uptake by the adjacent tissue (Speake *et al*, 1985), a considerable proportion (30-50%) of the fatty acid released may be carried away by the circulation and thus become generally available to tissues distal from the site of LPL action (Cryer, 1987; Karpe *et al*, 1992; Vernon and Clegg, 1984). Thus events occurring in the capillaries of the adipose tissue may result in DHA, again in the FFA form, being delivered to the brain. There is, however, no direct evidence that such a process may be occurring in the chick embryo; the high proportion of DHA in the plasma FFA fraction at certain developmental stages is compatible with both the "LPL theory" and the "preferential mobilisation theory" and of course, these two putative mechanisms are not mutually exclusive.

As a consequence of the substrate specificity of LPL, the remnant lipoproteins resulting from LPL action contain residual TG consisting of a very high proportion of DHA. Thus remnant uptake by the liver results in the hepatic accumulation of TG which is highly enriched in DHA. During the hatching period, this DHA is apparently translocated from TG to PL in the liver. It is tempting to suggest that this DHA-enriched PL is subsequently secreted from the liver in the form of VLDL and delivered to the brain as suggested by Scott and Bazan (1989), Li *et al* (1992) and Burdge and Postle (1994). The results of Tarugi *et al* (1994) indicate that the liver of the chick embryo synthesises and secretes apoB-containing lipoproteins. Thus it is conceivable that DHA transport is mediated by adipose tissue in the embryo and then by the liver after hatching. These concepts are summarised in Figure 4.3 and await testing by appropriate experiments.

4.5.3. The chick embryo as a model system for PUFA transport.

In order to be useful as a model system, in addition to being convenient for experimentation, it is desirable that work with the chick embryo produces concepts of more general application. A key concept from the present study is the potential role of adipose tissue as a mediator of DHA transport to the brain. In the human, maternal adipose tissue accumulates during early pregnancy and is extensively mobilised at the later stages to provide fatty acids for the fetus (Crawford *et al*, 1989). The possibility that DHA may be preferentially mobilised from the maternal adipose tissue to be released into the plasma in the FFA form, able to cross the placenta and be taken up by the fetal brain, may be worthy of investigation.

4.6.SUGGESTIONS FOR FUTURE WORK

A major outcome of this study is the highlighting of the role of adipose tissue in the mediation of DHA delivery to the embryonic brain. Future experiments to confirm the preferential mobilisation hypothesis may be suggested based on the incorporation and release of radioactively-labelled fatty acids by adipose tissue pieces or cells cultured *in vitro*. For example, following pre-incorporation of [^{14}C]16:0 and [^{3}H]DHA into the TG of adipose tissue *in vitro*, changes in the ^{14}C / ^{3}H ratio of both the tissue TG and the medium FFA during the subsequent chase incubation would provide a further test of the theory that DHA is preferentially mobilised from the tissue. The Hypothesis presented in Figure 4.3 is dependent on the view that the plasma FFA pool is the source of PUFA for the brain. Therefore a crucial test of this theory would depend on the provision of evidence that the brain of the chick embryo can take up FFA from the plasma. Experiments involving the injection of radiolabelled DHA into the main chorioallantoic vessel of the embryo and the subsequent measurement of incorporation of radioactivity into brain PL, and also *in vitro* experiments to investigate the uptake of labelled fatty acids by the brain cells in culture may be suggested.

In order for this work to have any practical value, it is necessary to extend these studies to mammals and in particular the human. For example, studies on the accretion of DHA into maternal adipose TG and the subsequent mobilisation of DHA to provide for the needs of the fetus would provide a background for the rational design of supplementation programmes to maximise the neurofunctional potential of the human infant.

Figure 4.2: Lipid uptake from the yolk and biochemical modifications within the yolk sac membrane (YSM). Yolk lipids are taken up by the YSM during embryonic development. It is not clear if all fatty acids incorporated are hydrolysed from their initial yolk lipid fraction (i.e. is AA hydrolysed from yolk PL form to be re-esterified in PL in YSM ?). DHA is hydrolysed from PL and reesterified in TG fraction. A proportion of 18:2 is hydrolysed and is elongated/desaturated to AA which is then mainly reesterified in PL. Cholesteryl esters are synthesised from yolk free cholesterol in YSM. The enzyme responsible for this esterification is acylCoA:cholesterol acyltransferase (ACAT). The newly formed lipid fractions are then assembled to very low density lipoprotein prior release in the embryonic circulation for subsequent delivery to the target organs. The fatty acid proportions are based on the situation at about day 12 of development.

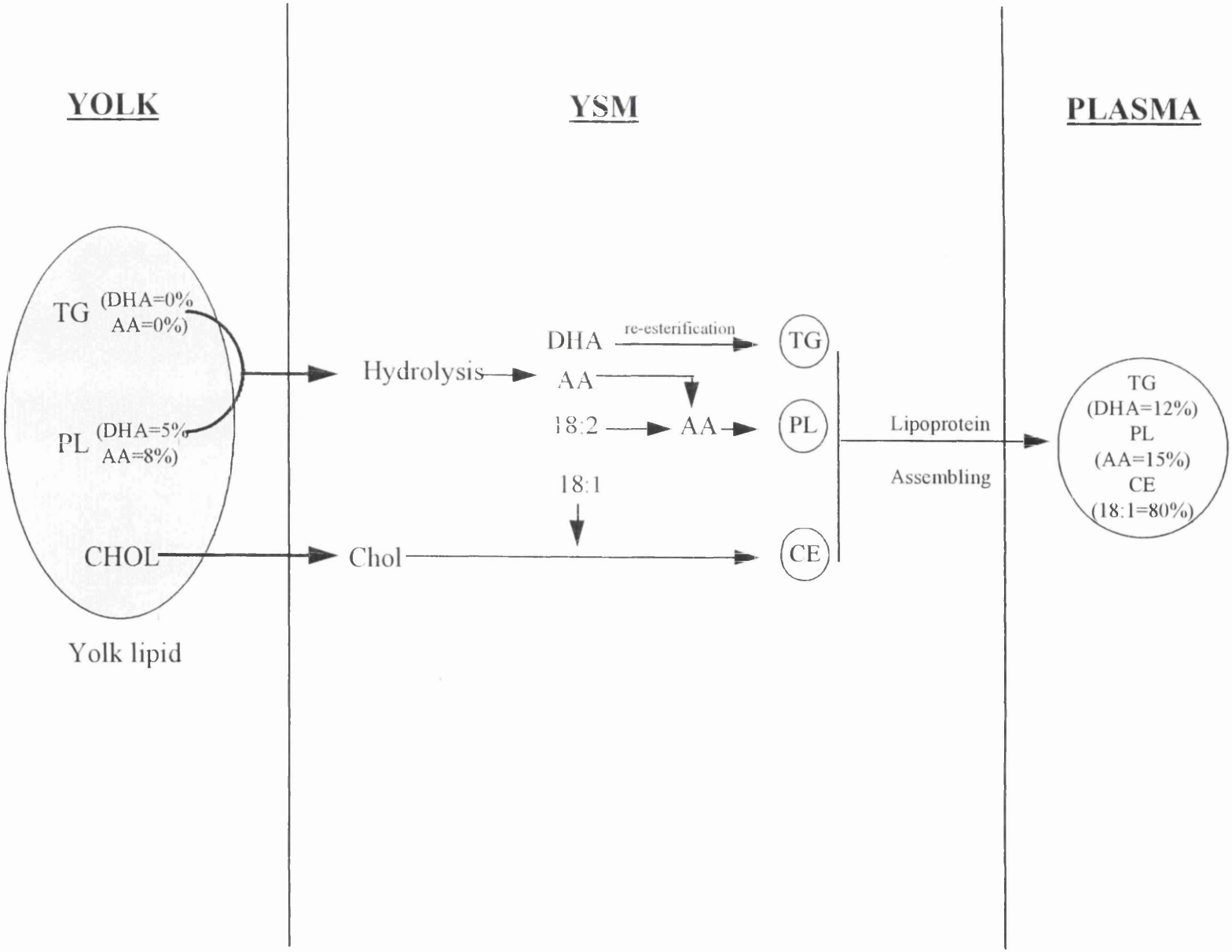
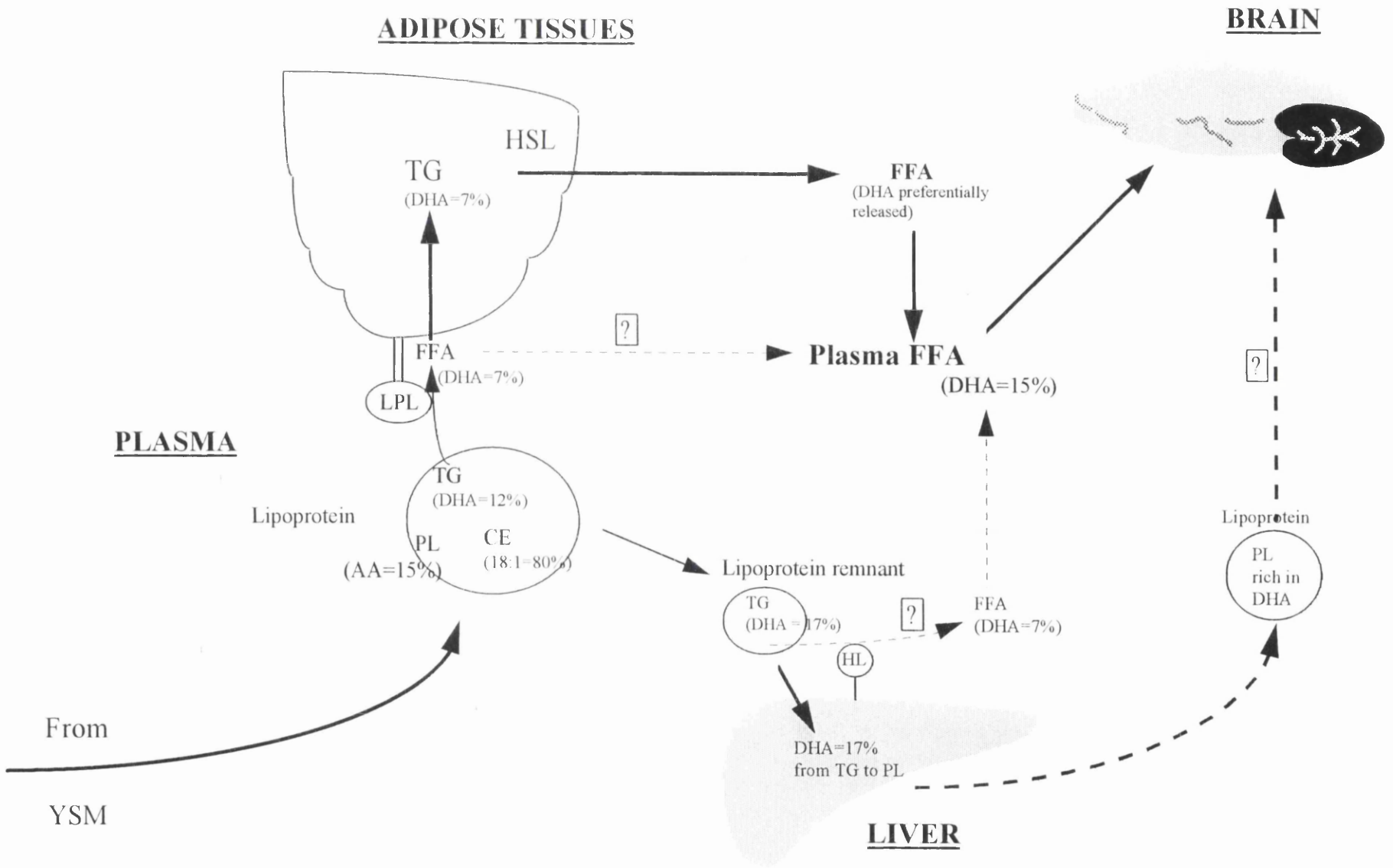

YOLK
YSM
PLASMA
TG (DHA=0% AA=0%)
PL (DHA=5% AA=8%)
CHOL
Yolk lipid
Hydrolysis
DHA
re-esterification
TG
AA
18:2
AA
PL
18:1
Chol
CE
Lipoprotein
Assembling
TG
(DHA=12%)
PL
(AA=15%)
CE
(18:1=80%)

Figure 4.3: A scheme for the delivery of DHA to the brain of the chick embryo. TG contained in lipoproteins are partially hydrolysed by lipoprotein lipase (LPL) situated mainly the blood vessels of adipose tissue. The major part of the FFA released in that way are then incorporated in adipose TG. It is unclear what proportion of this FFA remains in the plasma. The lipoprotein remnant is enriched in DHA since TG containing long chain PUFA are relatively resistant to LPL action; the remnant is taken up by the liver. The presence of a hepatic lipase (HL) in the embryonic liver may also release some DHA as FFA into the plasma. The present study has shown a preferential release of long chain PUFA from adipose TG which may then become available for the developing brain. A role for the liver in DHA delivery to the brain may also be considered since DHA is transfered from liver TG to PL and then possibly released into embryonic circulation in the lipoprotein form. The fatty acid proportions (% values) shown in the diagram are based on the values at around day 12 of development.


ADIPOSE TISSUES
BRAIN
HSL
TG
(DHA=7%)
FFA
(DHA preferentially released)
?
FFA
(DHA=7%)
LPL
Plasma FFA
(DHA=15%)
PLASMA
TG
(DHA=12%)
Lipoprotein
PL
(AA=15%)
CE
(18:1=80%)
Lipoprotein remnant
TG
(DHA=17%)
?
FFA
(DHA=7%)
HL
Lipoprotein
PL
rich in
DHA
DHA=17%
from TG to PL
From
YSM
LIVER

REFERENCES

Abumrad, N. A., Park, J. H. and Park, C. R. (1984). Permeation of long-chain fatty acid into adipocytes. *Journal of Biological Chemistry.* **259**:8945-8953.

Adams, P. B., Sinclair, A. J., Lawson, S. and Sanigorski A. (1995). Arachidonic acid to eicosapentaenoic acid ratio in blood correlates positively with clinical symptoms of depression. *Proceedings of the 2nd International Congress of the International Society for the Study of Fatty Acids and Lipids.* Bethesda, Maryland, USA.

Alberghina, M., Lupo, G. Anfuso, C. D. and Infarinato, S. (1994). Differential transport of docosahexaenoate and palmitate through the blood-retina and blood-brain barrier of the rat. *Neuroscience Letters.* **171**:133-136.

Alberghina, M., Lupo, G. Anfuso, C. D. and Moro, F. (1993). Palmitate transport through the blood-retina and blood-brain barrier of rat visual system during aging. *Neuroscience Letters.* **150**:17-20.

Alejandre, M. J., Marco, C., Ramirez, H., Segovia, J. and Garcia-Peregrin E. (1984). Lipid composition of brain myelin from normal and hyperphenylalaninemic chick embryos. *Comparative Biochemistry and Physiology.* **77B**:329-332.

Anderson, G. J. and Connor, W. E. (1988). Uptake of fatty acids by the developing rat brain. *Lipids*, **23**:286-290.

Anderson, G. J., Connor, W. E. and Corliss,, J. D.(1990). Docosahexaenoic acid is the preferred dietary n-3 fatty acid for the development of the brain and retina. *Pediatric Research.* **27**:89-97

Anderson, G. J., Connor, W. E., Corliss, J. D. and Lin, D. S. (1989). Rapid modulation of the n-3 docosahexaenoic acid levels in the brain and retina of the newly hatched chick. *Journal of Lipid Research.* **30**:433-441.

Anderson, G. J., Hohimer, A. R. and Willeke, G. B. (1993). Uptake of docosahexaenoic acid by microvessels from developing rat brain. *Life Sciences*, **53**:1089-1098.

Bacon, W. L., Brown, K. I. and Musser, M. A.(1973). Low density lipoproteins of chicken, turkey and quail egg yolk. *Poultry Science.* **52**:1741-1744.

Belfrage, P., Fredrikson, G., Stralfors, P. and Tornqvist, H. (1984). Adipose tissue lipases. In: *Lipases.* (Borgstrom, B. and Brockman, H. L. editors). 365-416. Elsevier. Amsterdam.

Bellairs, R. (1991). Overview of early stages of avian and reptilian development. In *Egg incubation: Its effects on embryonic development in birds and reptiles* (Deeming, D. C. and Ferguson, M. W. J., eds). 371-383. Cambridge University Press. Cambridge.

Bellairs, R. (1992). Fertilisation and early embryonic development in poultry. *Poultry Science.* **72**:874-881.

Ben-Zeev, O. Doolittle, M. H., Singh, N., Chang, C. H. and Schotz, M. C. (1990). Synthesis and regulation of lipoprotein lipase in the hippocampus. *Journal of Lipid Research.* **31**:1307-1313.

Billheimer, J. T., Tavani, D and Ness, W. R. (1981). Effect of a dispersion of cholesterol in Triton WR-1339 on Acyl CoA:cholesterol Acyltransferase in rat liver microsomes. *Analytical Biochemistry.* **111**:331-335.

Board, R. G. and Sparks, N. H. C. (1991). Shell structure and function in avian eggs. In *Egg incubation : Its effects on embryonic development in birds and reptiles* (Deeming, D. C. and Ferguson, M. W. J., editors). 71-86. Cambridge University Press. Cambridge.

Boren, J., Wettesten, M., Rustaeus, S., Anderson, M. and Olofsson, S. O. (1993). The assembly and secretion of apoB-100-containing lipoproteins. *Biochemical Society Transactions.* **21**:487-493.

Bourre, J. M., Faivre, A., Dumont, A., Nouvelot, A., Loudes, C., Puymirat, J. and Tixier-Vidal, A. (1983). Effect of polyunsaturated fatty acids of fetal mouse brain cells in culture in a chemically defined medium. *Journal of Neurochemistry.* **41**:1234-1242.

Brown, M. S., Kovanen, P. T. and Goldstein, J. L. (1981). Regulation of plasma cholesterol by lipoprotein receptors. *Science.* **212**:628-635.

The British Nutrition Foundation's Task Force (1992). *Unsaturated fatty acids: nutritional and physiological significance.* Chapman and Hall, London.

Burdge, G. C. and Postle, A. D. (1994). Hepatic phospholipid molecular species in the guinea pig : Adaptations to pregnancy. *Lipids.* **29**:259-264.

Burley, R. W., Evans, A. J. and Pearson, J. A. (1993). Molecular aspects of the synthesis and deposition of hens' egg yolk with special reference to low density lipoprotein. *Poultry Science.* **72**:850-855.

Cadigan, K. M., Heider, J. G. and Chang, T. Y. (1988). Isolation and characterization of Chinese-Hamster ovary cell mutants deficient in acyl-coenzyme-A-cholesterol acyltransferase activity. *Journal of Biological Chemistry.* **263**:274-282.

Calderon, R. O., Attema, B. and DeVries, G. H. (1995). Lipid composition of neuronal cell bodies and neurites from cultured dorsal root ganglia. *Journal of Neurochemistry.* **64**:424:429.

Carlson, S. E., Rhoades, P. G., Rao, V. S. and Goldgar, D. E. (1987). Effect of fish oil supplementation on the n-3 fatty acid content of red blood cell membranes in preterm infants. *Pediatric Research.* **21**:507-510.

Chapman, M. J. (1980). Animal lipoproteins : Chemistry, structure and comparative aspects. *Journal of Lipid Research.* **21**:789-853.

Cherian, G. and Sim, J. S. (1992). Preferential accumulation of n-3 fatty acids in the brain of chicks from eggs enriched with n-3 fatty acids. *Poultry Science.* **71**:1658-1668.

Cherian, G. and Sim, J. S. (1993). Net transfer and inncorporation of yolk n-3 fatty acids into developing Chick embryos. *Poultry Science.* **72**:98-105.

Christie, W. C. (1985). Rapid separation and quantification of lipid classes by high performance liquid chromatography and mass (light-scattering) detection. *Journal of Lipid Research.* **26**:507-512.

Cockburn, F. (1994). Neonatal brain and dietary lipids. *Archives of Disease in Childhood.* **70**:F1-F2.

Cohn, W., Gross, P., Grun, H., Loechleiter, F. , Muller, D. P. and Zulauf, M. (1992). Tocopherol transport and absorption. *Proceedings of the Nutrition Society.* **51**:81-115.

Connor, W. E., Lowensohn, R. and Hatcher, L. (1995). Enhanced ω3 fatty acid levels in the newborn infant by maternal ω3 fatty acid supplementation. *Proceedings of the 2nd International Congress of the International Society for the Study of Fatty Acids and Lipids.* Bethesda, Maryland, USA.

Coolbear, K. P., Berde C. B. and Keough K. M. W. (1983). Gel to liquid-crystalline phase transitions of aqueous dispersions of polyunsaturated mixed-acid phosphatidylcholines. *Biochemistry.* **22**:1466-1473.

Crawford, M. A. (1995). Essential fatty acid requirements during early development. *Proceedings of the 2nd International Congress of the International Society for the Study of Fatty Acids and Lipids.* Bethesda, Maryland, USA.

Crawford, M. A. (1993). The role of essential fatty acids in neural development: implications for perinatal nutrition. *American Journal of Clinical Nutrition.* **57**:703S-710S.

Crawford, M. A., Doyle, W., Williams, G. and Drury, P. J. (1989). *The role of fats in human nutrition* (Vergroesen, A. J. and Crawford, M. A., editors). 81-115. Academic Press, London.

Crawford, M. A., Hassam, A. G. and Stevens, P. A. (1981). Essential fatty acid requirements in pregnancy and lactation with special referentce to brain development. *Progress in Lipid Research.* **20**:30-40.

Cryer, A. (1987). Comparative biochemistry and physiology of lipoprotein lipase. In: *Lipoprotein lipase* (Borensztajn, J., editor). 277-327. Evener, Chicago.

Davison, A. N. (1965). Brain sterol metabolism. *Advances in Lipid Research.* **3**:171-196.

Darin-Bennett, A., Poulos, A. and White, I. G. (1974). The phospholipids and phospholipid-brain fatty acids and aldehydes of dog and fowl spermatozoa. *Journal of Reproduction and Fertility.* **41**:471-474.

Decuypere, E., Dewil, E. and Kuhn, E. R. (1991). The hatching process and the role of hormones. In: *Avian incubation* (Tullet S., editor). 239-256.

Delton, I., Gharib, A. Molière, P., Lagarde, M. and Sarda, N. (1995). Distribution and metabolism of arachidonic and docosahexaenoic acids in rat pineal cells. Effect of norepinephrine. *Biochimica et biophysica Acta.* **1254**:147-154.

Dhopeshwarkar, G. A. and Mead J. F. (1973). Uptake and transport of fatty acids into the brain and the role of the blood-brain barrier system. *Advances in Lipid Research.* **11**:109-142.

Dobbing, J. and Sands, J. (1979). *Early human development.* **3**:79-83. Cited by Burdge and Postle, 1994.

Dolphin, P. J. (1985). Lipoprotein metabolism and the role of apolipoproteins as metabolic programmers. *Canadian Journal of Biochemistry and Cell Biology.* **63**:850-869.

Dratz, E. A., Ryba, N., Watts, A. and Deese, A. J. (1987). Studies on the essential role of docosahexaenoic acid in visual excitation. *Investigations in Ophtalmology and Visual Sciences.* **11**:28-96.

Entenman, C., Lorenz, F. W. and Chaikoff, I. L. (1940). The lipid content of blood, liver and yolk sac of the newly hatched chick and the changes that occur in these tissues during the first month of life. *Journal of Biological Chemistry.* **133**:231-241.

Enslen, M., Milon, H. and Malnoe, A. (1991). Effect of low intake of *n-3* fatty acids during development on brain phospholipid fatty acid composition and exploratory behaviour in rats. *Lipids.* **26**:203-208.

Erickson, S. K., Shrewsbury, M. A., Brooks, C. and Meyer, D. J. (1980). Rat liver acyl-coenzyme A:cholesterol acyltransferase: its regulaton in vivo and some of its properties in vitro. *Journal of Lipid Research.* **21**:930-941.

Esko, J. D. and Raetz, C. R. H. (1983). Synthesis of phospholipids in animal cells. In: *The enzymes.* (Boyer, H. editor). **16**:207-253. New York. Academic Press.

Fain, J. N. and Garcia-Sainz, J. A. (1992). Adrenergic regulation of adipocyte metabolism. *Journal of Lipid Research.* **24**:945-966.

Farquharson, J., Cockburn, F., Patrick, W. A., Jamieson, E. C. and Logan, R. W. (1992). Infant cerebral cortex fatty acid composition and diet. *Lancet.* **340**:810-813.

Faulkner, A. and Jones, C. T. (1979). The distribution of enzyme and isoenzyme activities between parnchymal and haematopoietic cells in the liver of the foetal guinea pig. *Biochemical. Journal.* **178**:89-95.

Fenney, R. E. and Allison, R.G. (1969). *Evolutionary Biochemistry of Proteins.* Interscience, New-York.

Freeman, B. M. and Vince, M. A. (1974). *Development of the Avian Embryo.* General reference. Chapman and Hall, London.

Furuichi, T. and Mikoshiba, K. (1995). Inositol 1,4,5-triphosphate receptor-mediated Ca^{2+} signaling in the brain. *Journal of Neurochemistry.* **64**:953-959.

Gavino, V. C. and Gavino, G. R. (1992). Adipose hormone sensitive lipase preferentially releases polyunsaturated fatty acids from triglycerides. *Lipids.* **27**:950-954

Gazzah, N., Gharib, A, Croset, M., Bobillier, P., Lagarde, M. and Sarda, N. (1995). Decrease of brain phospholipid synthesis in free-moving n-3 fatty acid deficient rats. *Journal of Neurochemistry.* **64**:908-918.

Gilbert, A. B. (1971). *Physiology and Biochemistry of the Domestic Fowl.* **3**:1153-1208 (Bell, D. J. and Freeman,B. M.,eds) Academic Press, London.

Gilbert, A. B., and Pearson, R.A. (1983). *Nutritional Physiology of Farm Animals.* 243-260 (Rook, J. A. F. and Thomas P. C.,editors). Longmann, New-York.

Glen, A. I. M., Glen, E. M. T., Horrobin, D. F., Vaddadi, K. S., Spellman, M., Morse-Fisher, N., Ellis, K. and Skinner, F. S. (1994). A red cell membrane abnormality in a subgroup of schizophrenic patients: evidence for two diseases. *Schizophrenia Research.* **12**:53-61.

Goldstein, J. L. and Brown, M. S. (1977). The LDL pathway and its relationship to atherosclerosis. *Annual Review of Biochemistry.* **46**:897-930.

Goodridge,A. G. (1973). Regulation of fatty acids synthesis in isolated hepatocytes prepared from the livers of neonatal chicks (Hepatic concentrations of individual fatty acid and other chicks' metabolites). *Journal of Biological Chemistry.* **248**:1924-1931.

Green, P. and Yavin, E. (1993). Elongation, desaturation and esterification of essential fatty acids by fetal rat brain in vivo. *Journal of Lipid Research.* **34**:2099-2107.

Griffin, H. D. (1992). Manipulation of the egg yolk cholesterol : a physiologist's view. *World's Poultry Science Journal.* **48**:101-112.

Griffin, H. D., Perry, M. M. and Gilbert A. B. (1984). Yolk formation. *Physiology and biochemistry of the domestic fowl.* **5**:345-380. Academic Press, London.

Handelman, G. E., Boyles, J. K., Weisgraber, K. H., Mahley, R. W. and Pitas, R. E. (1992). Effects of apolipoprotein E, β-very low density lipoproteins, and cholesterol on the extension of neurites by rabbit dorsal root ganglion cells. *Journal of Lipid Research.* **33**:1677-1688.

Henderson, R. J. and Tocher, D. R. (1987). The lipid composition and biochemistry of freshwater fish. *Progress in Lipid Research.* **26**:281-347.

Hollenberg, C. H. and Angel, A. (1963). Relation of fatty acid structure to release and esterification of free fatty acids. *American Journal of Physiology.* **205**:909-912.

Holman, R. T., Johnson, S. B. and Hatch, T. F. (1982). Human linolenic acid deficiency. *American Journal of Clinical Nutrition.* **35**:617-623.

Hornstra, G., Al, M. D. M. and v Houwelingten, A. C. (1995). Essential fatty acids, pregnancy andpregnancy outcome. *Proceedings of the 2nd International Congress of the International Society for the Study of Fatty Acids and Lipids.* Bethesda, Maryland, USA.

Hunt, A. N., Bellhouse, A. F., Kelly, F.J. and Postle, A. D. (1991). Late gestation changes in rat-tissue phosphatidylcholine composition. *Biochemical Society Transactions.* **19**:111 S.

Husbands, D. H. R. and Brown, W. O. (1965). Sex differences in the composition and acetate incorporation into liver lipids of the adult fowl. *Comparative Biochemistry and Physiology.* **14**:445-451.

Innis, S. (1995). Nutritional programming by breastfeeding. *Proceedings of the 2nd International Congress of the International Society for the Study of Fatty Acids and Lipids.* Bethesda, Maryland, USA.

ISSFAL, International Society for the Study of Fatty Acids and Lipids, 2nd International Congress (June 7-10, 1995). *Fatty acids and Lipids From Cell Biology To Human Disease.* Bethesda, Maryland, USA.

Kanazawa, A., Miyazaura, T., Hirono, H., Hayashi, M. and Fujimoto, K. (1991). Possible essentiality of docosahexaenoic acid in Japanese monkey neonates: occurence in colostrum and low biosynthetic capacity in neonate brain. *Lipids.* **26**:53-57.

Karpe, F., Olivecrona, T., Walldius, G. and Hamsten, A. (1992). Lipoprotein lipase in plasma after an oral fat load- relation to free fatty acids. *Journal of Lipid Research.* **33**:975-984.

Koerker, D. J. and Fritz, I. B. (1970). Studies on the control of fatty acid oxidation in liver preparations from chick embryos. *Canadian Journal of Biochemistry.* **48**:418-424.

Kuhn, H. and Crawford, M. (1986). Placental essential fatty acid transport and prostaglandin synthesis. *Progress in Lipid Research.* **25**:345-353.

Lambson, R. O. (1970). An electron microscopic study of the endodermal cells of the yolk sac of the chick during incubation an d after hatching. *American Journal of anatomy.* **129**:1-20.

Langslow, D. R. (1972). The development of lipolytic sensitivity in the isolated fat cells of *Gallus domesticus* during the foetal and neonatal period. *Comparative Biochemistry and Physiology.* **43B**:689-701.

Laugharne, J. D. E., Mellor, J. E. and Peet M. (1995). Fatty acid and Schizophrenia. *Proceedings of the 2nd International Congress of the International Society for the Study of Fatty Acids and Lipids.* Bethesda, Maryland, USA.

Leyton, J., Drury, P. J. and Crawford, M. A. (1987). Differential oxidation of saturated and unsaturated fatty acids *in vivo* in the rat. *British Journal of Nutrition.* **57**:383-393.

L'hirondel, M., Chéramy, A., Godeheu, G. and Glowinski, J. (1995). Effects of arachidonic acid on dopamine synthesis, spontaneous release, and uptake in triatial synaptosomes from the rat. *Journal of Neurochemistry.* **64**:1406-1409.

Li, J., Wetzel M. G. and O'Brien P. J. (1992). Transport of n-3 fatty acids from the intestine to the retina in rats. *Journal of Lipid Research.* **33**:539-548.

Lin, D. S., Connor, W. E. and Anderson G. J. (1991). The incorporation of n-3 and n-6 fatty acids into the chick embryo from egg yolks having vastly different fatty acid compositions. *Pediatric Research.* **29**:601-605.

Lin, D. S., Connor, W. E., Wolf, D. P., Neuringer, M. and Hachey D. L. (1993). Unique lipids of primate spermatozoa : demosterol and DHA. *Journal of Lipid Research.* **34**:491-499.

Lucas, A., Morley, L., Cole, T. J., Lister, G. and Leeson-Payne, C. (1992). Breast milk and subsequent intelligence quotient in children born pre-term. *Lancet.* **34**:491-499.

Makrides, M., Neumann, M., Simmer, K., Pater, J. and Gibson, R. (June 10, 1995). Are long-chain polyunsaturated fatty acids essential nutrients in infancy? *Lancet.* **345**:1463-1468.

Mahley, R. W., Innerarity, T. L., Rall, S. C. and Weisgraber, K. H. (1984). Plasma lipoproteins: apolipoprotein structure and function. *Journal of Lipid Research.* **25**:1277-1294.

Meinhertz, H. (1963). Selective retention of oleic acid by adipose tissue in vitro. *Federation Proceedings*. **22**:375A.

Melin, T., Qi, C., Bengtsson-Olivecrona, G., Akesson, B and Nilsson, A. (1991). Hydrolysis of chylomicron polyenoic fatty acid esters with lipoprotein-lipase and hepatic lipase. *Biochimica et Biophysica Acta*. **1075**:259-266.

Moore, S. A., Sprecher H. and Spector, A. A. (1993). Cerebral endothelium synthesize docosahexaenoic acid (22:6n-3) from 24-carbon precursors. *Proceedings of the International Congress of the International Society for the Study of Fatty Acids and Lipids*. 93.

Moore, S. A., Yoder, E., Murphy, S, Dutton, G. R. and Spector, A. A. (1991). Astrocytes, not neurons, produce docosahexaenoic acid (22:6n-3) and arachidonic acid (20:4n-6). *Journal of Neurochemistry*. **56**:518-524.

Morley, R. (1995). Nutritional programming by breastfeeding. *Proceedings of the 2nd International Congress of the International Society for the Study of Fatty Acids and Lipids*. Bethesda, Maryland, USA.

Miyamoto K., Stephanides L. M. and Bernsohn J. (1967). Incorporation of [1-14C]linoleate and linolenate into polyunsaturated fatty acids of phospholipids of the embryonic chick brain. *Journal of Neurochemistry*. **14**:227-237.

Nakamura, H., Faludi, G. and Spitzer, J. J. (1966). Changes of individual free fatty acids (FFA) during glucose tolerance test. *Fedederation Proceedings*. **25**:209A.

Nelson, G. J. and Ackman, R. G. (1988). Absorption and transport of fat in mammals with emphasis on n-3 polyunsaurated fatty acids. *Lipids*. **23**:1005-1014.

Neuringer, M., Anderson, G. J. and Connor, W. E. (1988). The essentiality of N-3 fatty acids for the development and function of the retina and brain. *Annual Review of Nutrition*. **8**:517-541.

Neuringer, M., Connor, W. E., Lin D. S. and Barstad L. (1986). Biochemical evidence of *n-3* fatty acid deficiency in brain and retina of perinatal and juvenile rhesus monkeys. *Proceedings of the National Academy of Sciences of the U.S.A.*. **83**:4021-4025.

Nelson, G. J. and Ackman, R. G. (1988). Absorption and transport of fat in mammals with emphasis on n-3 polyunsaurated fatty acids. *Lipids.* **23**:1005-1014.

Neuringer, M., Anderson, G. J. and Connor, W. E. (1988). The essentiality of N-3 fatty acids for the development and function of the retina and brain. *Annual Review of Nutrition.* **8**:517-541.

Neuringer, M., Connor, W. E., Lin D. S. and Barstad L. (1986). Biochemical evidence of *n-3* fatty acid deficiency in brain and retina of perinatal and juvenile rhesus monkeys. *Proceedings of the National Academy of Sciences of the U.S.A..* **83**:4021-4025.

Neuringer, M., Connor, W. E., Petten C. V. and Barstad L. (1984). Dietary omega-3 fatty acid deficiency and visual loss in infant rhesus monkeys. *Journal of Clinical Investigations.* **73**:272-276

Newsholme, E. A. and Start, C. (1974). Adipose tissue and the regulation of fat metabolism. In: *Regulation and Metabolism.* (Wiley, J. & Sons, editors). 195-246.

Nilsson, A., Hjeltje, L. and Strandvik, B. (1992). Incorporation of dietary [^{14}C]arachidonic acid and [^{3}H]eicosapentaenoic acid into tissue lipids during absorption of a fish oil emulsion. *Journal of Lipid Research.* **33**:1295-1305.

Nilsson, A, Landin, B., Jensen, E. and Akesson, B. (1987). Absorption and metabolism of orally fed arachidonic and linoleic acid in the rat. *American Journal of Physiology.* **252**:G817-G824.

Nilsson, A and Melin, T. (1988). Absorption and Metabolism of orally fed AA and 18:2 in the rat *American Journal of Physiology.* **255**:G612-G618.

Nilsson-Ehle, P., Garfinkel, A. S. and Schotz, M. C. (1980). Lipolytic enzymes and plasma lipoprotein metabolism. *Annual Review of Biochemistry.* **49**:667-693.

Noble, R. C. (1986). Lipid metabolism in the chick embryo. *Proceedings of the Nutrition society.* **45**:17-25.

Noble, R. C. (1987). Lipid metabolism in the chick embryo : some recent ideas. *The Journal of Experimental Zoology Supplement.* **1**:65-73.

Noble, R. C. and Cocchi, M. (1989). The relationship between the supply and demand for essential polyunsaturated fatty acid during mammalian and avian embryonic development. *Research and Development in Agriculture.* **6**, 2:65-69.

Noble, R. C. and Cocchi, M. (1990). Lipid metabolism and the neonatal chicken. *Progress in Lipid Research.* **29**:107-140.

Noble, R. C., Mc Cartney, R. J. and Ferguson, M., W., J. (1993). Lipid and fatty acid compositional differences between eggs of wild and captive-breeding alligators (*Alligator mississippiensis*) : an association with reduced hatchability ? *Journal of Zoology* (London). **230**:639-649

Noble, R. C. and Moore, J. H. (1967a). The liver phospholipids of the developing chick embryo. *Canadian Journal of Biochemistry.* **45**:627-639.

Noble, R. C. and Moore, J. H. (1967b). The partition of lipids between the yolk and yolk sac membrane during the development of the chick embryo. *Canadian Journal of Biochemistry.* **45**:949-958.

Noble, R. C. and Ogunyemi, D. (1989). Lipid changes in the residual yolk and liver of the chick immediately after hatching. *Biology of the Neonate.* **56**:228-236.

Noble, R. C. and Ogunyemi, D. (1988). Absorbtion of residual yolk and liver lipid changes in the newly hatched chick. *Proceedings of the Nutrition society.* **47**:160A.

Noble, R. C., Shand, J. H., Connor, K. and Brown, D. (1986). Effect of parental age on fatty acid oxidation *in vitro* by the liver and heart of the chicken. *Proceedings of the Nutrition society.* **45**:103A.

Noble, R. C. and Shand, J. H. (1981). The placenta-its role in the relationships between the lipids of mother and foetus. *IRCS Journal of Medical Science.* **9**:174-177.

Noble, R. C. and Tullet S. G. (January 1989). How valuable is yolk fat to the chick embryo ? *International Misset Poultry.*

Nouvelot, A. Delbart, C. and Bourre J. M. (1986). Hepatic metabolism of dietary alpha linolenic in suckling rats, and its possible importance in polyunsaturated fatty acid uptake by the brain. *Annales of Nutrition and Metabolism.* **30**:316-323.

Olofsson, S. O.,Bjursell G., Bostrom, K., Carlsson, P., Elovson, J., Protter, A. A., Reuben, M. A. and Bondjers, G. (1987). Apolipoprotein B: Structures, biosynthesis and role in the lipoprotein assembly process. *Atherosclerosis.* **68**:1-17.

Olivecrona, T. and Bengtsson-Olivecrona, G. (1987). Milk lipoprotein lipase. In: *Lipoprotein lipase* (Borensztajn J. editor). 15-58. Evener, Chicago.

Olsen, R. E. and Henderson, R. J. (1989). The rapid analysis of neutral and polar marine lipids using double-development HPTLC and scanning densitomatry. *Journal of Experimental Marine Biology and Ecology.* **129**:189-197.

Onuma, Y. Masuzawa, Y. Ishima, Y. and Waku, K. (1984). Selective incorporation of docosahexaenoic acid in rat brain. *Biochimica et Biophysica Acta.* **793**:80-85.

Oscar, T. P (1995). Glucagon binding to receptors on the surface of chicken adipocytes. *Journal of Animal Sciences.* **73**:728-737.

Oscar, T. P (1992). Glucagon-induced desensitization of broiler adipocyte lipolysis. *Poultry Science.* **71**:1015-1021.

Oscar, T. P (1991). Glucagon-stimulated lipolysis of primary cultured broiler adipocytes. *Poultry Science.* **70**:326-332.

Palmer, B. D. and Guillette Jr, L. J. (1991). Oviductal proteins and their influence on embryonic development in birds and reptiles. *Egg incubation. Its effects on embryonic development in birds and reptiles.* 29-46 (Deeming, D. C. and Ferguson, M. W. J. eds). Cambridge University Press.

Pawlosky, R. Barnes, A and Salem Jr, N. (1994). Essential fatty acid metabolism in the feline: relationship between liver and brain production of long-chain polyunsaturated fatty acids. *Journal of Lipid Research.* **35**:2032-2040.

Peterson, C. L., Thompson, M. A. Martin, D. and Nadler, J. V. (1995). Modulation of glutamate and aspartate release from slices of hippocampal area CA1 by inhibitors of arachidonic acid metabolism. *Journal of Neurochemistry.* **64**:1152-1160.

Pitas, R. E., Boyles, J. K., Lee, S. H., Foss, D. and Mahley, R. W. (1987). Astocytes synthesise apolipoprotein E and metabolise apolipoprotein E-containing lipoproteins. *Biochimica et Biophysica Acta.* **917**:148-161.

Postle, A. D., Burdge, G. C. and Al, M. (1994). In: *World Review of Nutritional Dietetics.* (Simopoulos, A.P., editor) 75, Karger, Basel.

Pugh, E. and Sidbury Jr, J. B. (1971). Fatty acid oxidation in embryonic chick tissues. *Biochemica et Biophysica Acta.* **239**:376-383.

Raclot, T. and Groscolas, R. (1993). Differential mobilization of white adipose tissue fatty acids according to chain length, unsaturation, and positional isomerism. *Journal of Lipid Research.* **34**:1515-1526.

Rapoport, S. I. (1995). Use of radiolabelled fatty acids to examine brain phospholipid metabolism in vivo: relation to neuroplasticity and phospholipase activation. *Proceedings of the 2nd International Congress of the International Society for the Study of Fatty Acids and Lipids.* Bethesda, Maryland, USA.

Ragsdale Jr, C. W. and Brockes, J. P. (1991). Retinoic acid receptors and vertebrate limb morphogenesis: In: *Nuclear hormone receptors* (Parker M.G. editor). Academic Press Ltd., London.

Reinhart, M. P., Avart, S. J., Dobson, T. O. and Foglia, T. A. (1993). The presence and subcellular distribution of sterol carrier protein 2 in embryonic-chick tissues. *Biochemical Journal.* **295**:787-792.

Ridgway, N. and Dolphin, P. J. (1984). Lipoprotein lipase-mediator sequestration of long chain polyunsaturated triacylglycerols in serum LDL from normal and Hypothyroid rats. *Biochimica et Biophysica Acta.* **796**:64-71.

Rizzo, A. M., Galli, C. F., Montorfano, G. and Berra, B. (1995). Phospholipid distribution and fatty acid composition in different brain regions during chick embryo development. *Journal of Neurochemistry.* **64**:1728-1733.

Rodriguez de Turco, E. B., Gordon, G. A., Peyman G. A. and Bazan N. G. (1990). Preferential uptake and metabolism of docosahexaenoic acid in membrane phospholipids from rod and cone photoreceptor cells of human and monkey retinas. *Journal of Neuroscience Research.* **27**:522-532.

Rogers, L. J. (1995). Development of the central nervous system and activity patterns. In: *The development of brain and behaviour in the chicken.* (CAB International, editor). 4-17.

Roldan, E. R. S. and Hamilton, R. A. P. (1993). Diacylglycerol in the exocytosis of the mammalian sperm acrosome. *Biochemical Society Transactions.* **21**:284-289.

Romanoff, A. L. (1960). Structural and functional development. In: *The avian embryo* (Romanoff A.L., editor). 1072-1077. New-York. The Macmillan Company.

Romanoff, A. L. (1967). *Biochemistry of the avian embryo.* New-York. The Macmillan Company.

Romer, A. S. (1968). *The procession of life.* The World Publishing Co. New-York.

Rostas, J. (1991). Molecular mechanisms of neuronal maturation: a model for synaptic plasticity. In: *Neural and Behavioural Plasticity: The Use of The Domestic Chick as a Model.* (Andrew, R. J., editor). 177-211. Oxford University Press, Oxford. Cited by Rogers, 1995.

Rothlin, M. E., Rothlin, C. B. and Wendt, V. E. (1962). Free fatty acid concentration and composition in arterial blood. *American Journal of Physiology.* **203**:306-310.

Rubin, D. and Laposata, M. (1992). Cellular interactions between n-6 and n-3 fatty acids - A mass analysis of fatty acid elongation/desaturation, distribution among complex lipids and conversion to eicosanoids. *Journal of Lipid Research.* **33**:1431-1440.

Salem Jr., N., Olsson, N. U., Ward, G., Denkins, Y. and Pawlosky R. (1995). Alcoholism and essential fatty acids. *Proceedings of the 2nd International Congress of the International Society for the Study of Fatty Acids and Lipids.* Bethesda, Maryland, USA.

Sanders, T., A., B. (1988). Essential and *trans*-fatty acids in nutrition. *Nutrition Research Reviews.* 1:57-78.

Sarda, N. Gharib, A. Moliere, P., Grange, E., Bobillier, P. and Lagarde, M. (1991). Docosahexaenoic acid (cervonic acid) incorporation into different brain regions in the awake rat. *Neuroscience Letters.* **123**:57-60.

Schoentgen, F. Pignède, G., Bonanno, L. M. and Jollès, P. (1989). Fatty acid-binding protein from bovine brain. Amino acid sequence and some properties. *European Journal of Biochemistry.* **185**:35-40.

Scott, B. K. and Bazan, N. G. (1989). Membrane docosahexaenoate supplied to the developing brain and retina by the liver. *Proceedings of the National Academy of Sciences of the U.S.A..* **86**:2903-2907.

Sedlacek, J. (1972). Development of the optic afferent system in chick embryos. In: *Advances in Psychobiology*, (Newton, G. and Riesen, A, eds). **1**:129-170. Wiley, New-York.

Shand, J. H. and West, D. W. (1991a). Acyl CoA:cholesterol acyltransferase activity in the rat mammary gland : variation during pregnancy and lactation. *Lipids.* **26**:150-154.

Shand, J. H. and West, D. W. (1991b). Effects of an antiserum to rat growth hormone and bromocryptine on cholesterol-metabolising enzymes in the lacting mammary gland. *Journal of Endocrinology.* **128**:287-295.

Shand, J. H., West, D. W., McCartney, R. C., Noble, R. C. and Speake B. K. (1993). The esterification of cholesterol in the yolk sac membrane of the chick embryo. *Lipids.* **28**:621-625.

Shand, J. H., West, D. W., Noble, R. C. and Speake B. K. (1994). The esterification of cholesterol in the liver of the chick embryo. *Biochimica et Biophysica Acta.* **1213**:224-230.

Sinclair, A. J. (1975). Incorporation of radioactive polyunsaturated fatty acids into liver and brain of developing rat. *Lipids.* **10**:175-184.

Slack, J. M. W. (1991). *From Egg to Embryo.* 195-211. Cambridge University Press.

Sobue, K. and Nakajima, T. (1978). Changes in concentrations of polyamines and γ-aminobutyric acid and their formation in chick embryo brain during development. *Journal of Neurochemistry.* **31**:277-279. Cited by Rogers, 1995.

Speake, B. K., Noble, R. C. and McCartney, R. J. (1993). Tissue specific changes in lipid composition and lipoprotein lipase activity during the development of the chick embryo. *Biochimica et biophysica Acta.* **1165**:263-270.

Speake, B. K., Parkin, S. M. and Robinson, D. S. (1985). Lipoprotein lipase in the physiological system. *Proceedings of the 609th meeting of the Biochemical Society Transactions.* **13**:29-31.

Spector, R. (1988). Fatty acid transport through the blood-brain barrier. *Journal of Neurochemistry.* **50**:639-643.

Spitzer, J. J., Nakamura, H., Gold, M., Altschuler, H. and Lieberson, M. (1966). Correlation between release of individual fatty acids and fatty acid composition of adipose tissue. *Proceedings of the Society of Experimental Biology and Medicine.* **122**:1276-1279.

Stein, Y. and Stein, O. (1962). The incorporation and disappearance of fatty acids in the rat epididymal fat pad studied by the in vivo incubation technique. *Biochimica et Biophysica Acta.* **60**:58-71.

Tarugi, P., Nicolini, S., Marchi, L. Ballarini, G. and Calandra, S. (1994). Apolipoprotein B-100 production and cholesteryl ester content in the liver of developing chick. *Journal of Lipid Research.* **35**:2019-2031.

Taurog, A., Lorenz, F. W., Entenmann, C. and Chaikoff, I. L. (1944). The effect of diethylstilbestrol on the *in vitro* formation of phospholipids in the liver as measured with radioactive phosphorus. *Endocrinology*. **35**:483-487.

Thiès, F., Delachambre, M. C., Bentejac, M., Lagarde, M. and Lecerf, J. (1992). Unsaturated fatty acids esterified in 2-acyl-1-lysophosphatidylcholine bound to albumin are more efficiently taken up by the young rat brain than the unesterified form. *Journal of Neurochemistry*. **59**:1110-1116

Thiès, F., Pillon, C. Molière, P., Lagarde, M. and Lecerf, J. (1994). Preferential incorporation of *sn*-2 lysoPC DHA over unesterified DHA in the young rat brain. *American Journal of Physiology*. **36**:R1273-R1279.

Tocher, D. R., Mourente, G. and Sargent, J. R. (1992). Metabolism of [1-14C]docosahexaenoate (22:6n-3), [1-14C]eicosapentaenoate (20:5n-3) and [1-14C]linolenate (18:3n-3) in brain cells from juvenile turbot *Scophthalmus maximus*. *Lipids*. **27**:494-499.

Tuan, R. S. (1987). Mechanism and regulation of calcium transport by the chick embryonic chorioallantoic membrane. *Journal of Experimental Zoology*. Suppl. **1**:1-13.

Uauy, R. D., Birch, D. G., Birch, E. E., Tyson J. E. and Hoffman, D. R. (1990). Effect of dietary omega -3 fatty acids on retinal function of very low birthweight neonates. *Pediatric Research*. **28**:485-492.

Vernon, R. G. and Clegg, R. A. (1984). The metabolism of white adipose tissues *in vivo* and *in vitro*. In: *New perspectives in Adipose Tissue: Structure, Function and Development*. (Cryer, A. and Van, R, L. R., editors). 65-86.

Vieira, A. V. and Schneider, W. J. (1993). Transport and uptake of retinal during chicken oocyte growth. *Biochimica et Biophysica Acta*. **1169**:250-256.

Voss, A., Reinhart, M., Sankarappa, S. and Sprecher, H. (1991). The metabolism of 7,10,13,16,19 docosapentaenoic acid to 4,7,10,13,16,19-docosahexaenoic acid in rat liver is independent of 4-desaturase. *Journal of Biological Chemistry*. **266**:19995-20000.

Wainwright, P. E., Huang, Y. S., Bulman-Fleming, B., Mills, D. E. , Redden P. and McCutcheon, D. (1991). The role of *n-3* essential fatty acids in brain and behavioural development : a cross fostering study in the mouse. *Lipids*. **26**:37-45.

White, H. B. (1991). Maternal diet, maternal proteins and egg quality.. In: *Egg incubation: Its effects on embryonic development in birds and reptiles* (Deeming, D.C. and Ferguson, M. W. J. ed.). 1-15. Cambridge University Press, Cambridge.

Williams, R. J. Murphy, N., Glowinski, J. and Prémont, J. (1995). Glucose regulates glutamate-evoked arachidonic acid release from cultured striatial neurons. *Journal of Neurochemistry*. **65**:241-249.

Yaffei, N. and Noble, R. C. (1990). Further observations on the association between lipid metabolism and low embryo hatchability in eggs from yong broiler birds. *The Journal of Experimental Zoology*. **253**:325-329.

Yu, J. Y. L., Campbell, L. D. and Marquadt, R. R. (1976). Immunological and compositional patterns of lipoproteins in Chicken (*Gallus domesticus*) plasma. *Poultry Science*. **55**:1626-1631.

PUBLICATIONS ARISING SO FAR FROM THIS WORK

Full paper:

Maldjian, A., Farkas, K., Noble, R. C., Cocchi, M. and Speake, B. K. (1995). The transfer of docosahexaenoic acid from the yolk to the tissues of the chick embryo. *Biochimica et Biophysica Acta.* **1258**:81-89.

Short papers:

Maldjian, A., Noble, R. C. and Speake, B. K. (1993). The transfer of docosahexaenoic acid from the yolk to the tissues of the chick embryo. *Biochemical Society Transactions.* **21**:297S.

Maldjian, A., Speake, B. K. and Noble, R. C. (1995). How are polyunsaturated fatty acids delivered to the brain during embryonic development. *International Hatchery Practice.* In Press.

Speake, B. K., Noble, R. C., Maldjian, A. and Vajda, K. (1994). Cholesterol, docosahexaenoic acid and the embryo. *International Hatchery Practice.* **8**(5):25.